UNIVERSIDADE DE LISBOA
FACULDADE DE FARMÁCIA

ROLE OF GLUTATHIONE S-TRANSFERASE PI IN NEURONAL PROTECTION UNDER OXIDATIVE STRESS AND PROTEASOME INHIBITION

RELEVANCE TO PARKINSON'S DISEASE

Andreia Margarida Gonçalves das Neves Carvalho

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"Science, this very creative human endeavor to understand the nature of the reality that exists independently of ourselves, is impossible. By "impossible", I am not saying "very, very difficult", although it is that, as well."

Douglas R. Green, *Molecular Cell*, 2010, 40 (2): 176-8

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ABBREVIATIONS

γ-GT	γ-glutamyltranspeptidase
1-CysPrx	1-cysteine peroxiredoxin
4E-BP	eukaryotic translation initiation factor 4E-binding protein
AR	aldose reductase
ARE	antioxidant responsive element
AR-JP	autossomal recessive juvenile-onset parkinsonism
ASK1	apoptosis signal-regulating kinase 1
Cul3	cullin 3
DA	Dopaminergic
DCF-DA	2',7'-dichlorofluorescein diacetate
DNPH	2,4-dinitrophenylhydrazine
GFAP	glial fibrillary acidic protein
GPx	glutathione peroxidase
GR	glutathione reductase
Grx	glutaredoxin
GSH	glutathione (reduced form)
GSK-3β	glycogen synthase kinase 3β
GSSG	glutathione (oxidized form)
GST	glutathione S-transferases
GSTP	glutathione S-transferase pi
GSx	total free glutathione
GWAS	genome-wide association studies
HO-1	heme oxygenase 1
JNK	c-Jun N-terminal kinase
Keap1	Kelch ECH associating protein 1
ko	knockout
LB	Lewy bodies
LRRK2	leucine rich repeat kinase 2

MAPK	mitogen-activated protein kinase
MAPKK	MAPK kinase
MAPKKK	MAPKK kinase
MKK	MAPK kinase (same as MAPKK)
MPP+	1-methyl-4-phenyl-pyridinium
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
NF-κB	nuclear factor κB
NQO1	NAD(P)H: quinone oxidoreductase 1
Nrf2	nuclear factor-erythroid 2 (NF-E2)-related factor 2
OXPHOS	oxidative phosphorylation
PARIS	parkin-interacting substrate
PBS	phosphate buffer saline
PD	Parkinson's disease
PDI	protein disulfide isomerase
PGC-1α	PPARγ co-activator 1 alpha
PINK1	PTEN induced novel kinase 1
PPARγ	peroxisome proliferator-activated receptor gamma
PSH	protein sulfhydryl groups / protein cystein residues
PSSG	*S*-glutathionylated proteins
PTEN	phosphatase and tensin homolog
ROS	reactive oxygen species
SAPK	stress-activated protein kinase
SN	*substantia nigra*
SNCA	α-synuclein gene
SNpc	*substantia nigra pars compacta*
ST	*striatum*
TH	tyrosine hydroxylase
TNF-α	tumor necrosis factor alpha
TRAF2	TNF-α receptor associated factor 2
Ub	ubiquitin
UPS	ubiquitin-proteasome system
wt	wild-type

ABSTRACT

Although the molecular mechanisms underlying DA neuronal death in Parkinson's disease (PD) are still not completely understood, solid evidence has accumulated implicating mitochondrial dysfunction, oxidative stress and failure of proteolytic pathways in the pathogenesis of the disease.

Glutathione S-Transferase pi (GSTP), whose expression is regulated by the nuclear factor-erythroid 2-related factor 2 (Nrf2), has been shown to protect cells from reactive oxygen species by modulating *S*-glutathionylation of proteins, following oxidative and nitrosative stress, altering the levels of glutathione, and also by acting as a ligand-binding protein controlling the catalytic activity of kinases involved in cell-signaling pathways.

Experimental models of PD based on the use of the selective dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) are widely used as they recapitulate the neurological features of the disease, triggering a cascade of deleterious events that culminate with DA neuronal demise.

The main objective of the present work was to characterize the putative neuroprotective role of GSTP against MPTP-induced oxidative stress and to contribute to the elucidation of the role of proteasome inhibition in the MPTP Parkinson's disease mouse model. Moreover with this work we aimed to further investigate the molecular mechanisms underlying MPTP-induced DA neuronal death.

In the first part of this work we have demonstrated *in vivo* that GSTP is an endogenous regulator of the activity of c-Jun N-terminal kinase (JNK). We show that in mice brain, GSTP is able to bind JNK, through direct protein-protein interaction, inhibiting its activation, and consequently preventing its downstream effects on triggering cell death pathways. Moreover, we showed that GSTP knockout mice display increased susceptibility to MPTP neurotoxicity.

We have further characterized GSTP neuroprotective function by demonstrating for the first time that, *in vivo* GSTP potentiates Kelch ECH associating protein 1 (Keap1)

S-glutathionylation following MPTP administration, in mice brain. As Keap1 is the major endogenous regulator of Nrf2, Keap1 *S*-glutathionylation results in the subsequent Nrf2 pathway activation, and increased expression of GSTP, in a positive feedback regulatory loop.

Finally, as the ubiquitin-proteasome system (UPS) is also a critical player in several processes involved in the regulation of neuronal survival *vs* neuronal death pathways, and specifically in the regulation of the Nrf2-elicited antioxidant response, we investigated the modifications in UPS function, upon MPTP-induced oxidative stress and proteasome inhibition, in wild-type and GSTP knockout mice brain. We concluded that different components of the UPS have different susceptibilities to oxidative stress and, importantly GSTP knockout mice display increased susceptibility to UPS damage and inactivation upon MPTP-induced oxidative stress.

In conclusion, our results provided new insights into the molecular mechanisms involved in DA neuronal loss and we identified novel mechanisms involved in GSTP-elicited neuronal protection. Overall our results demonstrate important GSTP roles, other than detoxification, namely in the regulation of kinase signaling pathways and consequently in the modulation of cell death cascades, and in the potentiating of *S*-glutathionylation, thus enhancing antioxidant responses. Therefore the regulation of GSTP expression constitutes a promising therapeutic target in the development of novel therapeutic strategies for the treatment of neurodegenerative diseases and other diseases in which oxidative stress is an important factor of progression.

Keywords: Glutathione S-transferase pi – c-Jun N-terminal kinase – MPTP –Oxidative Stress – Ubiquitin Proteasome System – Parkinson’s Disease

RESUMO

A doença de Parkinson (PD) é uma doença neurodegenerativa caracterizada pela degenerescência progressiva dos neurónios dopaminérgicos da *substantia nigra pars compacta* (SNpc) e pela presença de inclusões citoplasmáticas constituídas por agregados proteicos, denominadas corpos de Lewy. A depleção de dopamina, resultante da morte dos neurónios dopaminérgicos, origina perda dos mecanismos de neurotransmissão entre a SNpc e o estriado, onde se situam os terminais nervosos destes neurónios, e conduz à incapacidade de controlo dos movimentos voluntários.

Embora se conheçam as características neuroquímicas e neuropatológicas da PD, a sua etiologia bem como os mecanismos moleculares subjacentes à morte dos neurónios dopaminérgicos não estão ainda completamente elucidados. No entanto, resultados de numerosos estudos apontam para um importante papel de factores como o stress oxidativo, a disfunção mitocondrial e a desregulação de vias de degradação proteica, na patogénese das formas familiar e esporádica da PD.

O desencadeamento da disfunção do metabolismo proteico, nas formas esporádicas da PD, poderá resultar do stress oxidativo mediado por espécies reactivas de oxigénio (ROS). A isoforma pi da Glutationo S-transferase (GSTP) é um enzima de destoxificação de fase II, que para além de catalisar a reacção de adição nucleofílica do glutationo reduzido a um elevado número de substratos, participando assim na eliminação das ROS, tem sido sugerido como protector em situações de stress oxidativo devido à sua capacidade de estabelecer interacções proteína-proteína, modulando vias de sinalização celulares.

Os modelos experimentais baseados na administração da neurotoxina 1-metil-4-fenil-1,2,3,6-tetrahidropiridina (MPTP) a ratinhos mimetizam diversas características neuropatológicas da PD desencadeando uma cascata de eventos causadores de danos celulares, que culmina com a degenerescência selectiva dos neurónios dopaminérgicos.

O presente trabalho teve como principal objectivo contribuir para a caracterização do potencial papel neuroprotector da GSTP, em resposta ao stress induzido pelo MPTP e

à inibição do proteossoma. Mais especificamente foram estudados os mecanismos moleculares subjacentes à perda dos neurónios dopaminérgicos em situações de stress oxidativo induzido pelo MPTP e investigado o papel da inibição do proteossoma neste modelo de PD.

Na fase inicial deste trabalho demonstrámos que a administração de MPTP a ratinhos C57BL/6 *wild-type* (wt) e GSTP *knockout* (ko) conduz à degenerescência selectiva dos neurónios dopaminérgicos da via nigro-estriado, com perda de densidade dos terminais nervosos no estriado e diminuição do número de neurónios dopaminérgicos no cérebro médio. Os nossos resultados mostraram que a degenerescência dos neurónios dopaminérgicos ocorre mais precocemente nos ratinhos GSTP ko do que nos wt, demonstrando que os ratinhos ko são mais susceptíveis à neurotoxicidade induzida pelo MPTP. Adicionalmente, observámos que o tratamento com MPTP conduz a um aumento da fosforilação da cinase N-terminal da c-Jun (JNK), bem como da sua actividade catalítica e consequentemente da fosforilação de c-Jun. A activação da cascata de sinalização da JNK despoleta vias de morte celular, pelo que os resultados obtidos indicam que esta será uma das vias envolvidas na morte dos neurónios dopaminérgicos induzida pelo MPTP. Os mecanismos moleculares subjacentes à regulação da actividade da JNK em condições de stress celular envolvem a activação de uma cascata de cinases bem como da regulação controlada de diversos repressores endógenos da JNK. Os nossos resultados demonstram que *in vivo* a GSTP actua como um regulador endógeno da resposta celular ao stress induzido pelo MPTP através da inibição directa da actividade da JNK, por interacção proteína-proteína.

O factor de transcrição Nrf2 é um dos principais sensores e reguladores celulares da resposta ao stress oxidativo e a sua actividade é regulada maioritariamente pela proteína Keap1, que encaminha o Nrf2 para degradação pelo proteossoma. A indução da expressão de enzimas de fase II, nomeadamente a GSTP pelo factor de transcrição Nrf2, constitui um potencial mecanismo de defesa celular contra o stress oxidativo induzido pelo MPTP. Numa segunda parte deste trabalho procurámos por um lado clarificar de que forma a inibição do proteossoma, adicionalmente ao stress induzido pelo MPTP, influenciava a regulação da actividade do Nrf2 e quais as consequências na activação da GSTP, e por outro investigar o papel da GSTP na regulação dos mecanismos moleculares activados nestas condições experimentais.

Os resultados obtidos demonstraram um aumento da formação de ROS bem como um aumento da acumulação de grupos carbonilo nas proteínas, indicando um aumento da quantidade de proteínas oxidadas no cérebro de ratinhos C57BL/6 wt tratados com MPTP e/ou com o inibidor do proteossoma, MG132. Verificou-se também um aumento da reactividade dos astrócitos e da microglia. Conjuntamente estes resultados sugerem uma activação inicial de vias de stress e inflamação, nas quais parece haver um efeito de sinergia resultante da administração simultânea dos dois compostos. Um dos marcadores de estado redox celular mais reconhecidos é o nível de glutationo intracelular, bem como o seu estado de oxidação. Os nossos resultados mostraram um decréscimo de glutationo, acompanhado de um aumento de actividade da peroxidase do glutationo e um decréscimo da actividade da reductase do glutationo, indicando um ambiente celular mais oxidante. No entanto não se verificou um aumento da forma oxidada livre do glutationo nestas condições, provavelmente porque o decréscimo observado nos níveis de glutationo livre se deve à sua conjugação com proteínas, visto que observámos um aumento dos níveis de proteínas *S*-glutationiladas nos ratinhos wt tratados com MPTP. Mais ainda, através de ensaios de co-imunoprecipitação verificámos a *S*-glutationilação específica da proteína Keap1, nos ratinhos wt mas não nos GSTP ko. A *S*-glutationilação conduz à dissociação do complexo entre a Keap1 e o Nrf2, evitando a degradação do Nrf2 pelo proteossoma e conduzindo à sua activação. Nos ratinhos wt tratados com MPTP, observámos ainda um aumento de expressão do Nrf2, bem como da sua translocação para o núcleo, indicativa da sua activação. Ainda de acordo com estes resultados, pudémos observar um aumento dos níveis de expressão da GSTP e da heme oxigenase (HO-1), ambas reguladas pelo Nrf2, nos ratinhos wt tratados com MPTP. No entanto, nos ratinhos ko não se verificou um aumento nos níveis de expressão para a HO-1.

Tendo em conta que a GSTP foi sugerida como sendo um dos catalisadores da reacção de *S*-glutationilação, estes resultados indicam que, em resposta ao stress induzido pelo MPTP, a GSTP catalisa a reacção de *S*-glutationilação da Keap1, levando à dissociação do complexo Keap1-Nrf2 e à activação do Nrf2. O Nrf2 por sua vez é translocado para o núcleo onde regula a expressão de vários genes, nos quais se incluem *GSTP* e *HO-1*. Nos ratinhos GSTP ko, a ausência de GSTP inviabiliza este mecanismo. No seu conjunto, estes resultados demonstram a existência de um mecanismo de regulação por *feedback* positivo, em que a GSTP ao ser activada em condições de stress, poderá por sua vez deslocar o equilíbrio da reacção de *S*-glutationilação no sentido da

glutationilação de diversas proteínas, nomeadamente da Keap1, levando à dissociação do complexo Keap1-Nrf2 e consequentemente a uma maior activação do Nrf2.

O conjunto dos nossos resultados permitiu demonstrar pela primeira vez a *S*-glutationilação da Keap1 *in vivo*, e consequente activação do Nrf2, revelando um novo mecanismo de protecção neuronal pela GSTP.

A disfunção das vias de degradação de proteínas é um dos factores envolvidos na degenerescência dopaminérgica na PD e em modelos experimentais. Por um lado a inibição das vias de degradação, nomeadamente do proteossoma, poderá causar a acumulação de proteínas danificadas por stress oxidativo, e por outro lado a acumulação de proteínas oxidadas poderá levar à sobrecarga e consequente inibição do proteossoma, criando assim uma potenciação cíclica dos danos oxidativos na célula. Na última parte deste trabalho procurámos clarificar de que forma a via da ubiquitina-proteossoma é alterada pela administração de MPTP e/ou MG132, e se existem diferenças entre ratinhos wt e GSTP ko no que diz respeito a esta via. Verificámos que, no cérebro de ratinhos wt e GSTP ko, diferentes componentes da via da ubiquitina proteossoma apresentam diferentes susceptibilidades ao stress induzido pelo MPTP ou ao tratamento com MG132. Mais importante, observámos que os ratinhos ko são mais susceptíveis à disfunção da via da ubiquitina proteossoma, após tratamento com MPTP, relativamente aos wt, apresentando menor capacidade de ubiquitinação e níveis de expressão do enzima de conjugação com a ubiquitina (E1) mais baixos. Uma vez que o aumento da proteólise pode de certo modo funcionar como um mecanismo antioxidante ao degradar proteínas oxidadas ou danificadas, impedindo a sua acumulação, o facto de os ratinhos GSTP ko terem défices nesta via pode contribuir para a sua maior susceptibilidade ao stress induzido pelo MPTP. Estes resultados sugerem que a GSTP pode também ter um papel na protecção/manutenção das vias de degradação proteica em condições de stress oxidativo.

Em conclusão, com a realização deste trabalho contribuímos para a elucidação dos mecanismos implicados na degenerescência dos neurónios dopaminérgicos induzida pelo MPTP e identificámos alguns dos mecanismos subjacentes à neuroprotecção conferida pela GSTP. A expressão alterada de GSTP foi já descrita como estando relacionada com diversas patologias humanas. Os resultados aqui apresentados mostram que além de um importante papel na destoxificação, *in vivo* a GSTP tem a capacidade de regular a actividade catalítica da JNK, afectando a modulação de vias de morte celular, bem como de catalisar a *S*-glutationilação de proteínas envolvidas na resposta antioxidante. Assim, a

regulação da expressão da GSTP revelou ser um importante alvo para o desenvolvimento de novas estratégias terapêuticas para o tratamento de doenças neurodegenerativas e outras doenças nas quais o stress oxidativo seja um importante factor de progressão.

Palavras chave: Glutationo S-transferase pi – cinase N-terminal da c-Jun – MPTP – Stress Oxidativo – Via Ubiquitina-Proteossoma – Doença de Parkinson

1

GENERAL INTRODUCTION

1.1 Parkinson's Disease (PD)

1.1.1 Etiology, Pathology and Genetics of PD

Parkinson's disease (PD) is the most prevalent neurodegenerative movement disorder and is estimated to affect approximately 1% of the population older than 65 years of age (Dauer and Przedborski 2003; Moore et al. 2005; Martin et al. 2011).

Clinical characteristic symptoms of PD include bradykinesia, resting tremor, rigidity and postural instability. The majority of the symptoms results primarily from the death of dopaminergic (DA) neurons in the *substantia nigra pars compacta* (SNpc), that project their terminals into the *striatum* (ST), leading to the subsequent depletion of dopamine and loss of signalling in the nigrostriatal pathway and producing motor dysfunction (Dauer and Przedborski 2003; Martin et al. 2011).

The pathological hallmarks of PD are the relative selective and progressive loss of DA neurons in the SNpc and the presence, in the surviving neurons, of intracytoplasmic protainaceous inclusions, denominated Lewy bodies (LB), and of dystrophic neurites, termed Lewy neuritis (Forno 1996; Moore et al. 2005).

Although the majority of PD cases are sporadic and its etiology incompletely understood, the discovery of genes associated with rare monogenic forms of the disease have provided important novel insights into the molecular pathways involved in the pathogenesis of the disease. Sporadic and monogenic forms of PD produce different pathological and clinical phenotypes, but they also share common features with particular focus on mitochondrial impairment (Moore et al. 2005; Martin et al. 2011). Recent genome-wide association studies (GWAS) have implicated genetic variability at two of the *loci* previously identified to be mutated in familial cases of PD, as significant risk factors for developing sporadic PD (Satake et al. 2009; Simon-Sanchez et al. 2009). Taken together these observations strongly suggest the convergence of different triggers in common signaling pathways central to DA neuronal degeneration in both familial and sporadic forms of PD (Moore et al. 2005; Martin et al. 2011).

The study of the genes linked to familial forms of PD and the processes that they regulate has given rise to a number of cellular and animal models that allowed the dissection of pathways involved in the disease and the identification of potential therapeutic targets (Dauer and Przedborski 2003; Dawson et al. 2010; Martin et al. 2011).

The first gene discovered to be mutated in familial PD was ~-*synuclein* (*SNCA*; (Polymeropoulos et al. 1996; Polymeropoulos et al. 1997; Kruger et al. 1998). Mutations in *SNCA* are known to cause autossomal dominant form of PD. So far, A53T, A30P and E46K missense mutations in *SNCA* (Polymeropoulos et al. 1997; Kruger et al. 1998; Zarranz et al. 2004) have been characterized and several studies have also established a link between familial forms of PD and duplications or triplications in the *SNCA* gene (Singleton et al. 2003; Moore et al. 2005; Gasser 2009; Nuytemans et al. 2010). Moreover, reinforcing the notion that α-synuclein also plays a role in sporadic PD, due to its accumulation in LB in sporadic PD brain (Spillantini et al. 1997; Spillantini et al. 1998; Trancikova et al. 2012), *SNCA* was one of the genes whose variability was pointed in GWAS to be associated with sporadic PD (Satake et al. 2009; Simon-Sanchez et al. 2009). Additionally, the link between sporadic PD and *SNCA* gene expression is supported by described polymorphisms in *SNCA* promoter region that might increase α-synuclein expression and evidence of reduced epigenetic silencing of *SNCA* (Gasser 2009; Jowaed et al. 2010). Taken together, these evidences support the hypothesis that a gain of function by α-synuclein underlies pathogenesis of both familial and idiopathic PD (Moore et al. 2005; Martin et al. 2011).

α-synuclein is a highly abundant protein throughout the mammalian brain and is enriched in presynaptic nerve terminals, where it can associate with lipid membranes and vesicular structures (Maroteaux et al. 1988; Dauer and Przedborski 2003; Moore et al. 2005). Despite its important role in both familial and sporadic forms of PD, the physiological function of α-synuclein remain elusive. Nevertheless, emerging evidence points toward a possible role in synaptic vesicle recycling and neurotransmitter release (Abeliovich et al. 2000; Kahle et al. 2002; Lotharius and Brundin 2002; Outeiro and Lindquist 2003; Burre et al. 2010; Nemani et al. 2010).

Wild-type α-synuclein has a natively unfolded structure but adopts α-helical configuration when bound to membranes, and in high concentrations or mutated forms it has a propensity to misfold into β-sheets and form toxic oligomers and aggregates (Conway et al. 1998; Conway et al. 2000; Caughey and Lansbury 2003). The selective vulnerability of DA neurons to mutations in SNCA may be related to the stabilization of toxic α-synuclein intermediary oligomeric species (protofibrils) by dopamine oxidation products (Conway et al. 1998; Conway et al. 2000). Reciprocally, α-synuclein protofibrils

can cause toxicity through permeabilization of synaptic vesicles, leading to dopamine leakage to the cytoplasm where it further generates oxidative stress.

Aging is one of the main risk factors of PD, and α-synuclein protein levels increase with aging in human *substantia nigra* (SN) (Li et al. 2004a). Moreover, aging could also contribute to α-synuclein toxicity due to the age-related accumulation of oxidative and nitrative α-synuclein modifications that promote aggregation (Giasson et al. 2000; Conway et al. 2001; Lee and Trojanowski 2006). Despite α-synuclein oligomerization seem to be a key pathogenic mechanism in PD, its precise downstream neurotoxic effects are not clear: it could be related to protein degradation pathways impairment, mitochondrial deficits, defective cellular trafficking or increased sensitivity to oxidative stress (Moore et al. 2005).

Leucine rich repeat kinase 2 (LRRK2) is a large multidomain protein with guanosine-5'-triphosphate (GTP)-regulated serine/threonine kinase activity (Funayama et al. 2002; West et al. 2005). Mutations in the GTPase/kinase region of LRRK2, usually leading to increased kinase activity, are associated with autosomal dominant PD and some are also associated with apparent sporadic cases of the disease (West et al. 2005; Satake et al. 2009; Simon-Sanchez et al. 2009; Dachsel and Farrer 2010). Mutations outside the catalytic domain of LRRK2 do not seem to be related with PD, possibly implicating the GTPase and kinase activity of LRRK2 as central to disease development.

LRRK2 is expressed throughout the normal brain, localizing to the endoplasmic reticulum, and it is present in LB in PD patients (Vitte et al. 2010). LRRK2 function is not yet clear, although it has been suggested that it could impact mitochondrial function through its putative substrate eukaryotic translation initiation factor 4E-binding protein (4E-BP), and it might interact with the microRNA pathway, regulating translation (Imai et al. 2008; Zid et al. 2009; Gehrke et al. 2010).

Additionally, LRRK2 could interact with α-synuclein promoting its aggregation. Notably, low expression levels of LRRK2 were detected in the SN of humans and rodents, compared to other brain regions, including the ST (Higashi et al. 2007b; Higashi et al. 2007a). This leads to the interesting possibility that mutated LRRK2 can promote α-synuclein aggregation, initiating a cascade of aggregation self-propagated from cell to cell, eventually transferred to the susceptible DA neurons. Moreover, in a line of LRRK2 ko mice there is α-synuclein accumulation and defects in autophagy-lysosomal pathway

and apoptosis (Tong et al. 2010) suggesting a possible role of LRRK2 in regulating protein degradation.

Mutations in the *parkin* gene were the first to be identified, as a genetic cause of autossomal recessive juvenile-onset parkinsonism (AR-JP) (Kitada et al. 1998). More than 100 mutations in *parkin* have been described in PD subjects resulting in a loss of parkin function (West and Maidment 2004; Hardy 2010; Nuytemans et al. 2010). Interestingly, inactivation of parkin may also play a role in sporadic PD, as misfolded forms of parkin, resulting from oxidative and nitrosative stress, have been described in the brain of sporadic PD patients (Winklhofer et al. 2003; Chung et al. 2004; Yao et al. 2004; LaVoie et al. 2005; LaVoie et al. 2007; Wong et al. 2007; Schlehe et al. 2008; Pilsl and Winklhofer 2012).

The *parkin* gene encodes for a predominantly cytosolic protein with a structure comprising an N-terminal ubiquitin-like domain, a central linker region and a catalytic C-terminal RING box domain constituted by two RING finger motifs separated by an in-between-RING domain. Parkin is an E3 ubiquitin ligase that mediates the covalent attachment of ubiquitin to protein substrates, either targeting them for proteasomal degradation or modulating their function in several cellular pathways (Shimura et al. 2000; Moore 2006).

As E3 ligases are important components of the cellular machinery that covalently tags target proteins with ubiquitin (Glickman and Ciechanover 2002), it was initially proposed that loss-of-function mutations in *parkin*, associated to loss of its E3 ligase activity, could cause impaired ubiquitination and proteasomal degradation leading to abnormal substrate accumulation and consequent neurotoxicity (Tanaka et al. 2004; Moore et al. 2005; Trancikova et al. 2012). However, despite parkin possesses a diverse array of putative substrates there is limited evidence of their accumulation both in the brain from AR-JP patients (Murakami et al. 2004) and in parkin knockout (ko) mice (Ko et al. 2005; Periquet et al. 2005; Ko et al. 2006), possibly because the ubiquitination of these substrates is related with other functions than degradation. On the other hand, proteomic analysis revealed decreases in proteins involved in mitochondrial functions, particularly oxidative phosphorylation (OXPHOS), and in protection against oxidative stress, in the ventral midbrain of parkin ko mice (Palacino et al. 2004). This was accompanied by decreases in mitochondrial respiratory capacity and age-dependent increases of oxidative damage.

The molecular mechanisms involved in neurodegeneration caused by parkin inactivation are not completely clear. Nevertheless, current knowledge point to a wide-ranging neuroprotective function of parkin, with particular emphasis on parkin role in the maintenance of mitochondrial function and dynamics. In fact, several studies demonstrated the neuroprotective role of parkin against mitochondrial toxins, excitotoxins, endoplasmic reticulum stress and proteotoxic stress in cellular and animal models (Imai et al. 2000; Petrucelli et al. 2002; Darios et al. 2003; Jiang et al. 2004; Henn et al. 2007; Bouman et al. 2011), involving several different pathways, such as nuclear factor-κB (NF-κB) (Henn et al. 2007; Sha et al. 2010), c-Jun N-terminal kinase (JNK) (Cha et al. 2005; Hasegawa et al. 2008) and phosphatidylinositol 3 kinase (PI3K) (Fallon et al. 2006; Yang et al. 2006). An interesting recent study has also uncovered a new role for parkin in the modulation of peroxisome proliferator-activated receptor gamma (PPARγ) co-activator-1α (PGC-1α) by a new parkin-interacting substrate, PARIS (Shin et al. 2011).

Mutations in the *phosphatase and tensin homolog* (*PTEN*)-*induced putative kinase* (*PINK1*) gene are the second most common genetic cause of autosomal recessive PD (Valente et al. 2001; Valente et al. 2004; Bonifati et al. 2005). *PINK1* gene encodes a serine/threonine protein kinase with an N-terminal mitochondrial targeting signal sequence, a transmembrane sequence, and a C-terminal kinase catalytic domain. Although it can be also found in the cytosol, PINK1 protein mainly resides in the outer mitochondrial membrane with its kinase domain oriented toward the cytosol suggesting that PINK1 substrates are cytosolic or mitochondrial-associated (Silvestri et al. 2005; Zhou et al. 2008b). Several *PINK1* mutations have been described and most PD-associated mutations are located within the kinase domain, compromising kinase activity, or interfering with protein stability (Valente et al. 2004; Moore et al. 2005; Deas et al. 2009; Thomas and Cookson 2009; Kawajiri et al. 2011; Pilsl and Winklhofer 2012).

Results from initial studies in cultured cells suggested that PINK1 could protect against mitochondrial dysfunction and apoptosis induced by proteasomal inhibition (Valente et al. 2004). It was also suggested that PINK1 could prevent mitochondrial dysfunction through phosphorylation of mitochondrial proteins, in response to cellular stress (Valente et al. 2004) although alternatively an inability to normally phosphorylate mitochondrial proteins could actually lead to mitochondrial dysfunction.

The potential protective antioxidant role of PINK1 is suggested by evidences of OXPHOS deficiency, increased lipid peroxidation and enhanced susceptibility to oxidative stress, reported in primary fibroblasts from PD patients with mutations in PINK1 (Hoepken et al. 2007). Recently the jointly role of PINK1 and parkin in mitochondrial quality control, and in particular in mitophagy, has been highlighted in several studies (Green et al. 2011; Vives-Bauza and Przedborski 2011; Youle and Narendra 2011; Novak 2012; Pilsl and Winklhofer 2012). The interplay between PINK1 and parkin will be further discussed in the context of mitochondrial function regulation.

1.1.2 Oxidative Stress and Mitochondrial Dysfunction in PD

Although the molecular mechanisms of DA neuronal demise are still unclear, solid evidence has accumulated implicating oxidative stress, mitochondrial dysfunction, failure of protein degradation pathways with accumulation of protein aggregates and microglial activation as critical players in the pathogenesis of PD (Dauer and Przedborski 2003; Jenner 2003; Moore et al. 2005; Henchcliffe and Beal 2008; Schapira and Jenner 2011; Exner et al. 2012; McCoy and Cookson 2012; Nunnari and Suomalainen 2012). All of these processes are associated with the generation of reactive oxygen species (ROS) that can cause damage to essential macromolecules, such as lipids, proteins, and DNA (Jenner 2003; van Muiswinkel and Kuiperij 2005; de Vries et al. 2008; Henchcliffe and Beal 2008; Nunnari and Suomalainen 2012).

Under physiological conditions the level of ROS formation is in equilibrium with the antioxidant capacity. However, when the production of ROS overwhelms the cellular antioxidant capacity, oxidative stress and subsequent damage occur (Jenner 2003; van Muiswinkel and Kuiperij 2005). The central nervous system is particularly sensitive to oxidative stress, owing to a high oxygen consumption and enrichment in polyunsaturated fatty acids, making it particularly vulnerable to lipid peroxidation.

Mitochondria have a diverse array of functions in the cell, including the production of ATP *via* OXPHOS, lipid biosynthesis and the maintenance of calcium homeostasis (Henchcliffe and Beal 2008; Exner et al. 2012; McCoy and Cookson 2012; Nunnari and Suomalainen 2012). However, mitochondria are also involved in the production of ROS through OXPHOS, and in the release of apoptotic factors (Exner et al. 2012; Martinez and Greenamyre 2012; Nunnari and Suomalainen 2012; Perier et al. 2012; Schapira 2012). Oxidative stress or electron transport chain inhibition can lead to

permeabilization of mitochondrial membrane by the action of pro-apoptotic factors, such as Bax and cytochrome *c* or by opening of the mitochondrial transition pore, leading to collapse of the mitochondria membrane potential and ultimately apoptotic cell death. Mitochondrial dysfunction also results in impaired energy metabolism that can render cells more vulnerable to excitotoxicity resulting from changes in the energy dependent cell membrane potential. Mitochondrial dysfunction, can thus be an important trigger of neuronal cell death in PD due to the increase in ROS and activation of apoptotic cell death pathways (Henchcliffe and Beal 2008; Exner et al. 2012; Martinez and Greenamyre 2012; McCoy and Cookson 2012; Nunnari and Suomalainen 2012; Perier et al. 2012; Schapira 2012).

The structure and localization of mitochondria are critical for their function and depend on highly regulated activities such as mitochondrial fission and fusion and motility (Henchcliffe and Beal 2008; Exner et al. 2012; Nunnari and Suomalainen 2012). The dynamic morphology change ensures the maintenance of a healthy mitochondrial pool and is also crucial for the appropriate subcellular localization of mitochondria. Mitochondrial motility enables the correct localization of these organelles to the sub-compartments in the cell with higher energy demand (Detmer and Chan 2007; Exner et al. 2012; McCoy and Cookson 2012). This is particularly relevant in neurons, where mitochondrial biogenesis takes place in the soma and the localization of mitochondria is then regulated by anterograde and retrograde transport, along microtubules and actin filaments within axons and dendrites and to the synapses (Frederick and Shaw 2007; MacAskill and Kittler 2010). The localization of mitochondria at the sites of synaptic activity ensures the maintenance of Ca^{2+} and energy homeostasis, fundamental to proper neuronal function (Detmer and Chan 2007; Exner et al. 2012; McCoy and Cookson 2012; Nunnari and Suomalainen 2012). The correct balance of the processes of mitochondrial fission and fusion as well as motility is thus extremely important in neurons, due to their high energy requirements and need for fine-tuned mitochondria localization, for the maintenance of synaptic transmission (Li et al. 2004b; MacAskill and Kittler 2010). Moreover, in neurons the modulation Ca^{2+} flux through the mitochondria is essential in neurogenesis and neuronal plasticity as well as in controlling neurotransmitter release (Detmer and Chan 2007; Exner et al. 2012; McCoy and Cookson 2012; Nunnari and Suomalainen 2012).

The most direct evidence for disrupted mitochondrial metabolism in PD has come from the finding that in *postmortem* brain tissue from sporadic PD patients the activity of complex I of the electron transport chain is decreased in the SN and frontal cortex (Schapira et al. 1989; Schapira et al. 1990; Schapira and Jenner 2011). Complex I impairment in PD could in fact be systemic, as decreased activity was also detected in platelets and defective OXPHOS was suggested to occur in skeletal muscle, thus being a promising target as a PD biomarker (Schapira et al. 1990; Haas et al. 1995; Penn et al. 1995; Parker et al. 2008).

The importance of mitochondrial dysfunction as a cause of PD was reinforced by the discovery of the neurotoxic effects of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP is a by-product of the chemical synthesis of a meperidine analog with potent heroin-like effects (Dauer and Przedborski 2003; Przedborski et al. 2004; Cannon and Greenamyre 2011). In the early 80's several drug addicts developed a profound parkinsonian syndrome after the intravenous use of street preparations of meperidine analogs which were contaminated with MPTP (Langston et al. 1983). Since then, MPTP systemic administration has been one of the most best characterized and widely used experimental animal models of parkinsonism faithfully replicating the majority of the behavioral and pathological hallmarks of PD (Przedborski et al. 2000; Przedborski et al. 2001; Dauer and Przedborski 2003; Przedborski et al. 2004; Cannon and Greenamyre 2011; Martinez and Greenamyre 2012).

After systemic administration MPTP, which is highly lipophilic, rapidly crosses the blood-brain barrier (Saporito et al. 2000) and once in the brain is metabolized, in glial cells, into its active toxic metabolite, 1-methyl-4-phenylpyridinium (MPP^+) (Markey et al. 1984; Przedborski et al. 2000; Przedborski et al. 2001; Przedborski et al. 2004) (Figure 1.1). After being released to the extracellular space, MPP^+ selectively accumulates in DA neurons, due to its high affinity for the plasma membrane dopamine transporter (Javitch et al. 1985; Przedborski et al. 2000; Przedborski et al. 2004; Przedborski and Ischiropoulos 2005; Cannon and Greenamyre 2011). Once inside DA neurons, MPP^+ primarily concentrates in the mitochondria where it blocks the complex I of the electron transport chain, disrupting the electron flux. The consequent production of ROS and decreased ATP synthesis are thought to be the main mechanisms that cause DA neuronal death (Nicotra and Parvez 2002; Przedborski et al. 2004; Henchcliffe and Beal

2008; Cannon and Greenamyre 2011; Exner et al. 2012; Jackson-Lewis et al. 2012; Martinez and Greenamyre 2012).

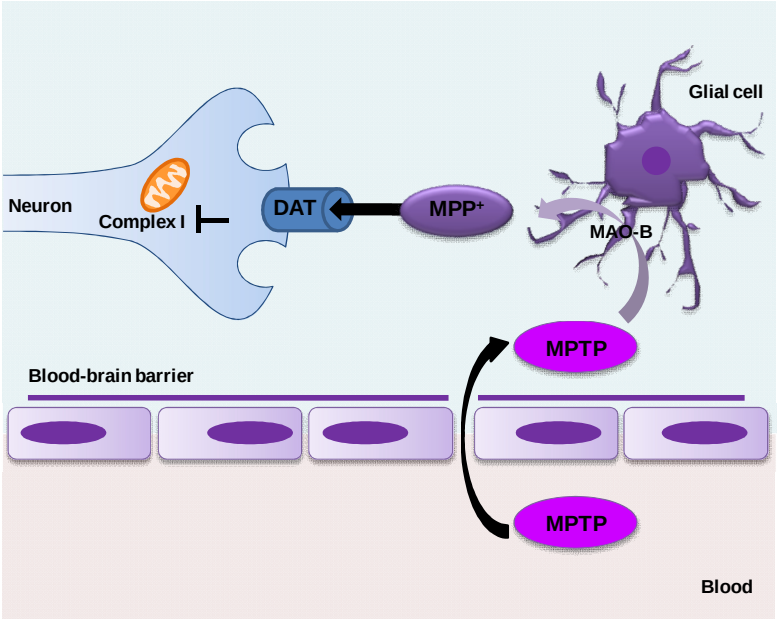

Figure 1.1 MPTP's mode of action. The neurotoxin MPTP is a highly lipophilic molecule and thus, after systemic administration it crosses the blood-brain barrier. Once in the brain, the pro-toxin is metabolized in glial cells, through a reaction catalyzed by the monoamine-oxidase B (MAO-B) enzyme, into its active toxic metabolite, MPP^+. After being released into the extracellular space, MPP^+ selectively enters and concentrates into dopaminergic neurons (DA), due to its high affinity for the plasma membrane dopamine transporter (DAT). Once inside DA neurons, MPP^+ primarily concentrates in the mitochondria where it blocks the complex I of the electron transport chain, disrupting the electron flux, with consequent production of ROS and decreased ATP synthesis. *Adapted from Dauer and Przedborski 2003.*

MPTP, as other well-known mitochondrial toxins (e.g. rotenone, paraquat), induce a cascade of deleterious events that recapitulate most of the neuropathological features of PD in laboratory animals (Dauer and Przedborski 2003; Przedborski et al. 2004; Cannon and Greenamyre 2011; Jackson-Lewis et al. 2012; Martinez and Greenamyre 2012). The fact that exposure to an acute neurotoxin like MPTP leads to active neurodegeneration that prolonged for years after the initial insult, as testified by astrogliosis and microgliosis in the surroundings of surviving dopaminergic neurons, is suggestive of an ongoing neurodegenerative process long after the acute toxicant exposure (Langston et al. 1999; McGeer and McGeer 2008; Cannon and Greenamyre 2011).

The advances in PD genetics in last years have further confirmed the important role of mitochondrial dysfunction in the pathogenesis of PD, since the protein products of several PD-associated genes including SNCA, LRRK2, parkin and PINK1, localize to the

mitochondria under certain conditions and modulate its function. Several animal models based on the expression and mutation of these genes present dysfunctional mitochondria (Henchcliffe and Beal 2008; Schapira and Jenner 2011; Exner et al. 2012; Martinez and Greenamyre 2012).

Studies on the subcellular localization of α-synuclein in rodent brain revealed that besides its association with vesicles and membranous structures, α-synuclein can specifically be associated with mitochondrial membrane, or be transported to the mitochondria under conditions of increased cellular stress or cytosolic acidification (Li et al. 2007; Cole et al. 2008; Exner et al. 2012). Furthermore the mitochondrial localization of α-synuclein in *postmortem* PD brains was reported and suggested to be dependent of a putative mitochondrial targeting sequence in the α-synuclein amino-terminus (Devi et al. 2008).

α-synuclein induced toxicity might be explained by its deregulation of the mitochondrial physiological activity. In fact, mitochondrial dysfunction and morphologic abnormalities have been reported in several transgenic models overexpressing α-synuclein (reviewed by Trancikova 2012 (Trancikova et al. 2012)). The first description of mitochondrial dysfunction in a transgenic mouse model of α-synuclein was reported on a mouse line expressing the A53T mutated α-synuclein under the mouse PrP promoter (Martin et al. 2006). Moreover, α-synuclein overexpression, in cellular models, causes mitochondrial depolarization and impairs mitochondrial activity and is associated with increased oxidative stress, decreased proteasome activity and increased susceptibility to mitochondria-dependent apoptosis (Hsu et al. 2000; Tanaka et al. 2001). In general, the overexpression of both wild-type and mutated forms of α-synuclein results in mitochondrial dysfunction with concomitant increase of oxidative stress that could be associated to oxidative modifications of mitochondrial proteins, increased calcium and nitric oxide levels and apoptosis (Tanaka et al. 2001; Parihar et al. 2008).

Studies in toxin-induced animal models reported that α-synuclein overexpression increases the susceptibility of neurons to mitochondrial toxins. Specifically studies with mutant α-synuclein transgenic mice demonstrated an increased susceptibility to MPTP-induced DA neuronal degeneration, accompanied by mitochondrial dysfunction and accumulation of α-synuclein aggregates (Song et al. 2004; Nieto et al. 2006; Yu et al. 2008). On the other hand α-synuclein ko mice display resistance to several toxins

including MPTP (Dauer et al. 2002; Klivenyi et al. 2006; Thomas et al. 2011). Therefore, α-synuclein effects potentially occur downstream of MPTP-induced mitochondrial dysfunction, and affect specifically DA neurons, since α-synuclein ko mice were shown to exhibit normal sensitivity to neurotoxicity induced by an MPTP analog that selectively targets serotonergic and noradrenergic neurons (Thomas et al. 2011). This DA neuronal selective vulnerability to mitochondrial dysfunction is further reinforced by the fact that, even though rotenone is highly lipophilic and enters any type of cell, chronic infusion of rotenone in rats elicits selective DA cell loss (Betarbet et al. 2000).

Mutated LRRK2 exhibits increased kinase activity and the phosphorylation of 4E-BP, a putative LRRK2 substrate, leads to its inactivation. Overexpression of 4E-BP suppresses DA neuron pathology in flies with dLRRK2 mutation; and increased 4E-BP activity, by treatment with rapamycin, or loss of dLRRK suppressed degeneration of DA neurons in PINK1 and parkin mutants (Imai et al. 2008; Tain et al. 2009). A possible role for 4E-BP activity in regulating mitochondrial function has been suggested (Zid et al. 2009) that could explain its protective effects following loss of PINK1 and parkin and might be relevant for PD pathogenesis. Moreover, 4E-BP binds to the eukaryotic translation initiation factor (eIF4E) that enhances mitochondrial activity due to the translational upregulation of nuclear encoded mitochondrial gene expression (Zid et al. 2009). Thus, aberrant phosphorylation of 4E-BP by LRRK2 could impact mitochondrial function and neuronal survival under stress conditions.

Parkin neuroprotective effects were suggested to be mediated through the regulation of a number of mitochondria-associated processes namely, mtDNA transcription/replication, OXPHOS, mitochondria morphology and biogenesis and by conferring protection against mitochondria-dependent cell death (Moore 2006).

Studies with parkin-mutant leukocytes (Muftuoglu et al. 2004) and fibroblasts (Mortiboys et al. 2008) derived from PD patients, showed alterations in mitochondrial morphology and activity and have highlighted a role for parkin in mitochondrial function. Moreover, in proliferating cells parkin was demonstrated to be associated with mitochondrial transcription factor A, enhancing its transcriptional activity, and thus regulating mtDNA transcription and replication (Kuroda et al. 2006).

Interestingly, recently Shin *et al* demonstrated that the conditional inactivation of parkin in the SN of adult mice induces the progressive degeneration of DA neurons through a mechanism involving the downregulation of PGC-1α (Shin et al. 2011). In this

study the authors demonstrated that PARIS is regulated by parkin *via* ubiquitin proteasome system. In the absence of parkin, in conditional parkin ko mice, PARIS accumulates and binds to PGC-1α promoter repressing the expression of this master regulator of mitochondrial biogenesis and leading to progressive loss of DA neurons (Rodgers et al. 2008; Wenz 2009; Scarpulla 2011; Shin et al. 2011). Therefore, parkin-mediated PARIS degradation increases the expression of PGC-1α downstream targets. As PGC-1α regulates mitochondrial biogenesis and energy metabolism this mechanism likely has tremendous impact on mitochondrial function and energy homeostasis regulation.

Parkin and PINK1 have been suggested to have complimentary or parallel functions in mitochondrial function and oxidative stress-related pathways. In fact, several studies supported a contribution of oxidative stress related with parkin and PINK1 deficits to PD pathology. For instance, PINK1 or parkin deficient *Drosophila* have reduced resistance to exogenous oxidative stress sensors and in these models neuronal death could be prevented by antioxidants overexpression (Whitworth et al. 2005; Wang et al. 2006). A plausible cause for endogenous oxidative stress in these flies is elevated ROS generation due to mitochondrial dysfunction. Remarkably, parkin overexpression was able to completely rescue these phenotypes in PINK1-deficient flies, whereas PINK1 overexpression did not prevent them in parkin mutant flies (Clark et al. 2006; Park et al. 2006; Yang et al. 2006). The observation, in these studies, that parkin and PINK1 mutant flies present similar phenotypes, indicate that these two proteins act in a common molecular pathway, in which PINK1 is likely to be upstream (Clark et al. 2006; Park et al. 2006; Yang et al. 2006).

Recent evidences suggest that upon loss of mitochondrial membrane potential, PINK1 selectively recruits parkin inducing its translocation to the mitochondria (Narendra et al. 2008; Okatsu et al. 2010; Vives-Bauza et al. 2010). Under physiological conditions, PINK1 is present at low levels on the outer mitochondrial membrane, however, in damaged mitochondria, PINK1 becomes stabilized and accumulates in the outer mitochondrial membrane (Jin et al. 2010; Narendra et al. 2010), triggering the translocation of parkin, which normally resides in the cytoplasm, to the depolarized or damaged mitochondria (Kim et al. 2008; Sha et al. 2010). This process has been suggested to be of key importance to the degradation of damaged mitochondria through mitophagy (Vives-Bauza et al. 2010; Vives-Bauza and Przedborski 2011). Multiple

mitochondrial proteins and factors involved in the processes of mitochondrial fission and fusion and in mitophagy have been recently proposed to be substrates of the E3 ligase activity of parkin (Poole et al. 2008; Gegg et al. 2010; Geisler et al. 2010; Ziviani et al. 2010; Chan et al. 2011; Glauser et al. 2011; Rakovic et al. 2011; Wang et al. 2011). A role for the PINK1/parkin pathway in mediating the autophagic clearance of damaged mitochondria is supported by the accumulation of abnormal large, swollen mitochondria in PINK1, and parkin ko fly models. Therefore, the PINK1/parkin pathway may modulate mitochondrial dynamics through the regulation of fission and fusion events and through promotion of mitophagy. Mutations in PINK1 or parkin seem to interfere with parkin recruitment, substrate ubiquitination, and mitophagy (Detmer and Chan 2007; Devi et al. 2008; Rakovic et al. 2011). This could implicate deficits in parkin and PINK1 dependent mitochondrial turnover as contributors in the pathogenesis of PD.

1.1.3 Protein Degradation Pathways and PD

Many neurodegenerative diseases, including PD, are characterized by the accumulation of misfolded, damaged protein deposits in affected brain regions, thus suggesting the involvement of proteolytic pathways dysfunction in the pathogenesis of these diseases (Taylor et al. 2002; Ciechanover and Brundin 2003; McNaught and Olanow 2003). The two major intracellular protein degradation systems are the ubiquitin-proteasome system (UPS) and the autophagy-lysosomal system. Neurons, as highly metabolically active, post-mitotic cells, are especially vulnerable to the accumulation of defective proteins, and this may account for the frequency with which conformational diseases affect the nervous system. In most cases these proteinaceous deposits are composed of ubiquitin conjugates, suggesting a failure in the clearance of proteins targeted for proteasomal degradation. It has also been suggested that autophagy plays a role in the initiation or progression of some neurodegenerative diseases (McCray and Taylor 2008), due to the observed accumulation of autophagic vacuoles, in neurons from affected brain regions in a number of neurodegenerative diseases, including Alzheimer's disease and Parkinson's disease (Anglade et al. 1997; Nixon et al. 2005). However, in many cases the exact role of protein degradation pathways dysfunction in the progression of the disease and whether it is a cause or a consequence of other damaging events is controversial (Ciechanover and Brundin 2003).

Autophagy is an evolutionary conserved process whose primary task in lower organisms is the maintenance of metabolic homeostasis in the face of changing nutrient availability. In this catabolic process, intracellular components, such as organelles and proteins, are delivered to the lysosomal compartment for degradation. Autophagy is primarily responsible for the degradation of long-lived proteins and maintaining amino acid pools in the setting of chronic starvation, although its contribution to the degradation of defective proteins may equal that of the UPS (Cuervo 2006; Nedelsky et al. 2008). Recent advances have demonstrated that in addition to its known role in the cellular adaptation to nutritional stress, autophagy also participates in a surprisingly diverse array of functions, including development, cell differentiation and tissue remodeling, intrinsic and extrinsic immunity, host-to-pathogen response, mechanisms related to cell death and cell survival in response to intracellular and extracellular stresses, and the removal of toxic or altered components from inside the cells (Cuervo 2004; Mizushima 2005; Nedelsky et al. 2008).

Autophagy has been frequently seen as a neuronal killer due to the massive accumulation of autophagic vacuoles reported in dying neurons. However, two reports from independent groups, have provided conclusive genetic evidence for a crucial role of autophagy in the continuous turnover of cytoplasmic contents in neurons, and shown that this process is essential for cell survival (Hara et al. 2006; Komatsu et al. 2006). Furthermore, it has also been demonstrated that pharmacological activation of autophagy reduces the toxicity that is associated with expression of the altered proteins in different neurodegenerative disorders, due to the involvement of this process in the clearance of protein aggregates (Ravikumar et al. 2004; Rubinsztein et al. 2005).

In light of these results, the upregulation of autophagy that is observed in many neurodegenerative disorders was proposed to be a defensive mechanism that is activated to compensate for the blockage of other proteolytic systems (Cuervo 2004; Iwata et al. 2005). In line with this, in the particular case of PD, the neuronal protein α-synuclein, can be degraded by the UPS, macroautophagy and chaperone mediated autophagy (Webb et al. 2003; Cuervo et al. 2004; Shin et al. 2005; Bandhyopadhyay and Cuervo 2007). Moreover, recently it was reported that α-synuclein is degraded by the proteasome, however when its expression is increased autophagy is required to clear it from the cell (Ebrahimi-Fakhari et al. 2011). Although the UPS and autophagy were long viewed as independent and parallel degradation systems, several lines of evidence have developed

suggesting that the UPS and autophagy are interrelated processes, functioning in a coordinated and complementary manner that becomes critical in times of cellular stress, and implicating the autophagic pathway as a compensatory mechanism for degrading misfolded proteins when the UPS is impaired, protecting against the accumulation of toxic species in neuronal cells (Bandhyopadhyay and Cuervo 2007; Martinez-Vicente and Cuervo 2007; Massey et al. 2008; Nedelsky et al. 2008).

The UPS is the major non-lysosomal protein degradation pathway within cells and the primary proteolytic complex responsible for the elimination of damaged and misfolded intracellular proteins often formed upon oxidative stress challenge (Ciechanover and Brundin 2003; Olanow and McNaught 2006; Schwartz and Ciechanover 2009). Inhibition of UPS plays a key role in mediating cellular toxicity in neurodegenerative diseases in general, and particularly in PD (McNaught and Jenner 2001; Ciechanover and Brundin 2003; Olanow and McNaught 2006; Schwartz and Ciechanover 2009). Furthermore, the UPS was one of the pathways that have been highlighted by genetic studies as being involved in PD as mutations in genes encoding for UPS-associated proteins were shown to cause certain forms of PD. Indeed, mutations in *parkin*, an E3 ubiquitin ligase (Kitada et al. 1998), and ubiquitin C-terminal hydrolase-L1 (UCH-L1) (Leroy et al. 1998), a deubiquitinating enzyme, were found in patients with familial PD (Dawson and Dawson 2003; Martin et al. 2011). Moreover, impairment of UPS has been demonstrated by the decrease in proteasomal enzyme activities in the SN of patients with idiopathic PD (McNaught and Jenner 2001). Interestingly, parkin, which is mutated in certain forms of PD (Kitada et al. 1998; Shimura et al. 2001) is the E3 responsible for UPS-mediated α-synuclein degradation, and it is also involved in clearance of damaged mitochondria through mitophagy (Green et al. 2011; Vives-Bauza and Przedborski 2011). Moreover, mutations in parkin may affect different domains of this E3 ligase with some of them decreasing the affinity for the 19S regulatory particle of the proteasome (Safadi et al. 2011). In addition, mutations in the Rpn10 subunit of the 19S regulatory particle of the proteasome were shown to increase the risk for PD (Wahl et al. 2008).

Furthermore, one of the pathological hallmarks of PD is the presence of Lewy bodies, protainaceous inclusions that stain for α-synuclein and ubiquitin, among other proteins (Forno 1996; Dauer and Przedborski 2003). Aside being degraded by the UPS and different types of autophagic processes, α-synuclein has also been suggested to be

degraded non-canonically by the 20S proteasome in an ubiquitin-independent manner (Tofaris et al. 2001). Interestingly, it has been reported that depletion of the proteasome subunit Rpt2 leads to accumulation of α-synuclein and development of Lewy-like inclusions (Bedford et al. 2008).

Some years ago, Olanow and collaborators (McNaught et al. 2004) have proposed that proteasome inhibition could provide a new model for the study of PD, however this turned out to be polemic as whereas some groups were able to reproduce the model and observe at least some of the features observed by that authors (Schapira et al. 2006; Zeng et al. 2006) other groups failed to reproduce the results (Bove et al. 2006; Kordower et al. 2006). Surprisingly it was even demonstrated that in some experimental conditions proteasome inhibition can induce increased expression of neuroprotective factors (Yew et al. 2005). Moreover, a protective effect of proteasome inhibitors against dopaminergic cell death was shown in a rat PD model using the neurotoxin 6-hydroxydopamine (Inden et al. 2005). It was also reported that proteasome inhibition blocked MPP^+-induced dopaminergic cell death in primary neuronal cultures of rat mesencephalon (Sawada et al. 2004).

1.2 Oxidative Stress and Antioxidant Response

1.2.1 The Nrf2-ARE system: main regulator of the antioxidant response

Although the human brain constitutes only 2% of the body weight, brain cells use around 20% of all the oxygen consumed (Dringen and Hirrlinger 2003), rendering them especially prone to generate high levels of ROS by OXPHOS. The amount of ROS formed is counter-balanced by an antioxidant defense mechanism consisting of endogenous antioxidant enzymes whose transcription is tightly regulated by the nuclear factor-erythroid 2 (NF-E2)-related factor 2 (Nrf2) (Ishii et al. 2002; Lee and Johnson 2004; de Vries et al. 2008).

Nrf2 is able to bind the *cis*-acting antioxidant responsive element (ARE), also known as electrophile responsive element (EpRE), in the promoter region of target genes that encode phase II detoxification enzymes and antioxidant proteins such as glutathione S-transferases (GST), NAD(P)H:quinone oxidoreductase 1 (NQO1) and heme oxygenase (HO-1), regulating their transcription (Ishii et al. 2002; Nioi et al. 2003; Lee and Johnson 2004; Zhang 2006; Nguyen et al. 2009; Hayes et al. 2010; Baird and Dinkova-Kostova

2011). The Nrf2-ARE signaling pathway is one of the major mechanisms in the cellular defense against oxidative stress and highlighting the importance of this pathway Nrf2 knockout mice were shown to have decreased expression of both constitutive and inducible levels of phase II enzymes and endogenous antioxidants, thus displaying increased susceptibility to several xenobiotics and are resistant to chemoprotective compounds (Chan and Kan 1999; Enomoto et al. 2001; Kwak et al. 2001; Ramos-Gomez et al. 2001; Chanas et al. 2002; Rangasamy et al. 2004; Calkins et al. 2005; Khor et al. 2006; Xu et al. 2006; Zhang 2006).

1.2.1.1 Nrf2: structure and regulation

Nrf2 is a basic leucine zipper (bZIP) transcription factor belonging to the Cap 'n' Collar (CNC) subfamily of regulatory proteins (Motohashi et al. 2002), and was first cloned and characterized by its ability to bind the NF-E2/activator protein-1 (AP-1) enhancer elements in the promoter of the β-globin gene (Moi et al. 1994) and later it was shown to bind to the *cis*-acting regulatory element, ARE, in the promoter regions of several cytoprotective genes (Itoh et al. 1995; Itoh et al. 1997; Kobayashi et al. 2002; Sykiotis and Bohmann 2008).

Nrf2 is a ubiquitously expressed protein with 605 amino acids and is organized in six functional domains named Neh 1-6 (Nrf2-ECH homology; Figure 1.2) (Itoh et al. 1999). The Neh1 domain contains the CNC-type bZIP, which is necessary for DNA binding and heterodimerization with other transcriptional partners such as the small Mafs (Itoh et al. 1995; Itoh et al. 1997; Marini et al. 1997; Itoh et al. 1999; Katsuoka et al. 2005; Nioi et al. 2005). Additionally within this domain there is a functional nuclear localization signal (NLS) (Jain et al. 2005). The N-terminal Neh2 domain contains seven lysine residues, allowing ubiquitin conjugation, and in fact it was identified by deletion analysis as the negative regulatory domain of Nrf2 activity, through which Nrf2 is driven to proteasomal degradation (Itoh et al. 1999; Zhang et al. 2004). Moreover, by yeast two-hybrid screen, using Neh2 as a bait, the Kelch-like ECH associated protein 1 (Keap1) was identified by Yamamoto and colleagues as the repressor of Nrf2 (Itoh et al. 1999). Thus, the Neh2 is the Keap1 binding site in Nrf2. The C-terminal Neh3 domain is the binding site for CHD6, a member of the chromo-ATPase/helicase DNA binding protein family, which functions as transcriptional co-activator to promote the transcription of ARE-dependent genes (Nioi et al. 2005). The Neh4 and Neh5 are two independent

transactivation domains that act synergistically to interact with another transcriptional co-activator CREB-binding protein (CBP) (Katoh et al. 2001). The Neh6 domain is a serine-rich domain that controls the Keap1-independent negative regulation of Nrf2 (McMahon et al. 2004).

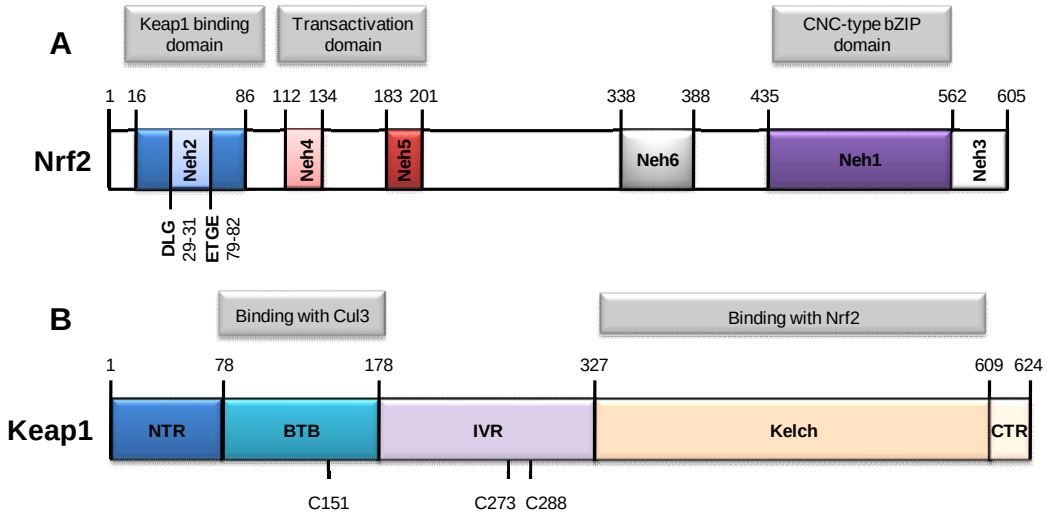

Figure 1.2 Nrf2 and Keap1 protein domains. A) Nrf2 is organized in six functional domains, here depicted, and named Neh 1-6. Neh1 domain contains the CNC-type bZIP, which is necessary for DNA binding and heterodimerization with transcriptional partners such as the small Mafs. Neh2 contains the DLG and ETGE motifs, through which Nrf2 binds to its transcriptional repressor Keap1. The C-terminal Neh3 domain is the binding site for CHD6, which functions as transcriptional co-activator to promote the transcription of ARE-dependent genes. Neh4 and Neh5 are two independent transactivation domains that act synergistically to bind the transcriptional co-activator CBP. The Keap1-independent negative regulation of Nrf2 is controlled by the Neh6 domain. **B)** Keap1, the key regulator of Nrf2 activity, is organized in three main domains. Near the N-terminal (NTR) localizes the BTB domain, responsible for Keap1 dimerization and for binding to Cul3 through the C151 cysteine residue. Adjacent to the C-terminal region (CTR) is the Kelch domain, through which Keap1 interacts with the Neh2 domain of Nrf2. And finally, between these two domains is the intervening region (IVR) that contains the C273 and C288 cysteine residues with important roles in redox sensing. *Adapted from Baird and Dinkova Kostova 2011*.

Keap1, the key regulator of Nrf2 activity, is a 624-amino acid protein organized in three main domains (Figure 1.2): an N-terminal BTB (Broad-Complex Tramtrack and Bric-a-Brac) dimerization domain, also responsible for the binding to Cullin 3 (Cul3) that contains cysteine C151, critical to the regulation of Keap1-Nrf2 binding; a cysteine-rich Intervening Region (IVR), that contains the cysteines C273 and C288 with crucial roles in the Keap1 redox sensing function; and a C-terminal Kelch domain with 6 Kelch repeats, through which Keap1 interacts with the Neh2 domain of Nrf2 (Zipper and Mulcahy 2002; Zhang and Hannink 2003; Zhang 2006; Baird and Dinkova-Kostova 2011). A key distinguishing feature of Keap1 is the high number of cysteine residues, 25 in murine and

rat Keap1 and 27 cysteine residues within the human homologue. Furthermore, by virtue of their location adjacent to basic amino acids, 10 of these cysteines are predicted to be highly reactive. The positive charge of these amino acids lowers the pK_a of the neighbouring cysteine thiol group, stabilizing the thiolate anion and therefore maintaining the cysteines in a reactive state (Snyder et al. 1981; Baird and Dinkova-Kostova 2011). Soon it was realized that these reactive cysteine residues in Keap1 played crucial roles in redox sensing and Nrf2 regulation (Dinkova-Kostova et al. 2002). The analysis of Keap1 cysteines suggested that C151, C273 and C288 are important for the function of Keap1. However, from the different results obtained in different studies, it was concluded that Keap1 contains multiple sensors (Kobayashi et al. 2009) and that different classes of inducers may react with these Keap1 cysteine residues in different ways (Wang et al. 2008). Hayes and colleagues reported that C151 and C288 each comprise discrete sensors, whilst a third sensor is formed by H225, C226 and C613 and each of these sensors is specific for certain types of inducers (McMahon et al. 2010).

Mutation analysis of the IVR domain showed that substitution of C273 or C288 with either serine or alanine rendered Keap1 unable to repress Nrf2 activity under basal conditions, suggesting that these residues are important for the repression of Nrf2 by Keap1 under basal conditions and that their modification may reduce the rate of ubiquitination and degradation of Nrf2 (Zhang and Hannink 2003; Levonen et al. 2004; Wakabayashi et al. 2004). *In vivo* experiments using transgenic mice expressing either C273A or C288A Keap1 mutants confirmed that these residues are required for repression of Nrf2 under basal conditions (Yamamoto et al. 2008). Different studies suggested that Nrf2 stabilization is not only dependent on the disruption of Nrf2-Keap1 binding but could also depend on the destabilization of the Keap1-Cul3 E3 ligase complex. Interestingly, the C151 residue in the BTB domain of Keap1 was shown to be important for the interaction with Cul3 (Zhang et al. 2004; Rachakonda et al. 2008).

Structural analysis also support these data: the fact that modifications of the IVR-residing C273 and C288 may affect the Nrf2 binding in the Kelch domain is supported and might be explained by the structural proximity of the two domains (Ogura et al. 2010). On the other hand, the assigned position of C151, far away from the Kelch domain, is more consistent with its modification being more likely to affect the interaction between Keap1 and Cul3, rather than Nrf2 (Ogura et al. 2010).

Despite the fact that Keap1 is undoubtedly the major regulator of Nrf2 activity, at the molecular level, the regulatory mechanisms involved in Keap1-mediated Nrf2 repression are not fully understood (Lee and Johnson 2004; Kobayashi et al. 2006; Zhang 2006; de Vries et al. 2008; Baird and Dinkova-Kostova 2011). Several models have been proposed for the regulation of the Keap1-Nrf2 pathway.

One of the first proposed mechanisms postulates that under basal conditions Nrf2 was sequestered in the cytoplasm by binding to a Keap1 dimer which in turn was anchored to actin. Oxidative stress or chemoprotective/inducer compounds lead to the disruption of the complex, releasing Nrf2 which translocates to the nucleus and enhances the expression of cytoprotective genes (Itoh et al. 1999; Dinkova-Kostova et al. 2002). However, succeeding reports showed that this was not the case for all kinds of inducing stimulus, suggesting that the model should be adjusted (Zhang et al. 2004; Eggler et al. 2005). Data from several subsequent studies showed that certain compounds induced an increase in Nrf2 half-life leading to its accumulation. Interestingly, inhibiting the proteasome resulted in similar achievements and Nrf2 was shown to be a target of ubiquitination, strongly suggesting that Nrf2 can be regulated at the level of protein stabilization (Sekhar et al. 2000; Itoh et al. 2003; McMahon et al. 2003; Nguyen et al. 2003; Stewart et al. 2003). Even though it was already established that Nrf2 activity was regulated by its stability and inactivation of Keap1 was required for stabilization the precise regulatory mechanism was still unknown. By that time four independent groups reported that Keap1 was able to bind Cul3, through its BTB domain, functioning as an adaptor protein for the ubiquitination of Nrf2 (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004; Furukawa and Xiong 2005). It was then suggested that Nrf2, as an unstable protein, is constantly being degraded by the proteasome. Keap1 appears to promote Nrf2 ubiquitination in a constitutive manner through the Cul3-dependent pathway. In this case, activation of Nrf2 was suggested to be dependent on mechanisms that increase its stability, leading to its accumulation in the cell (Nguyen et al. 2003; Nguyen et al. 2009).

Taken together these results given rise to the most commonly accepted mechanism of regulation of Nrf2 activity (Figure 1.3). According to this model under basal conditions Nrf2 is sequestered in the cytoplasm by Keap1, which in association with the Cul3-Rbx1 forms an E3 ubiquitin ligase complex that targets Nrf2 for ubiquitination and proteasomal degradation (Cullinan et al. 2004; Kobayashi et al. 2004; Zhang et al. 2004; Furukawa

and Xiong 2005). By this way Keap1 represses Nrf2 transactivation activity and accelerates its degradation through the proteasome. This quenching interaction maintains low basal expression of Nrf2-regulated genes. Oxidative and electrophilic challenges disrupt the complex by modification of reactive cysteine residues of Keap1 thereby leading to Nrf2 stabilization and allowing it to escape proteasomal degradation and translocate to the nucleus. Once in the nucleus Nrf2 binds to the ARE and recruits the general transcription machinery for expression of ARE-regulated genes, including several cytoprotective genes that enhance cell survival (Lee and Johnson 2004; Kobayashi et al. 2006; Zhang 2006; Kensler et al. 2007; Baird and Dinkova-Kostova 2011).

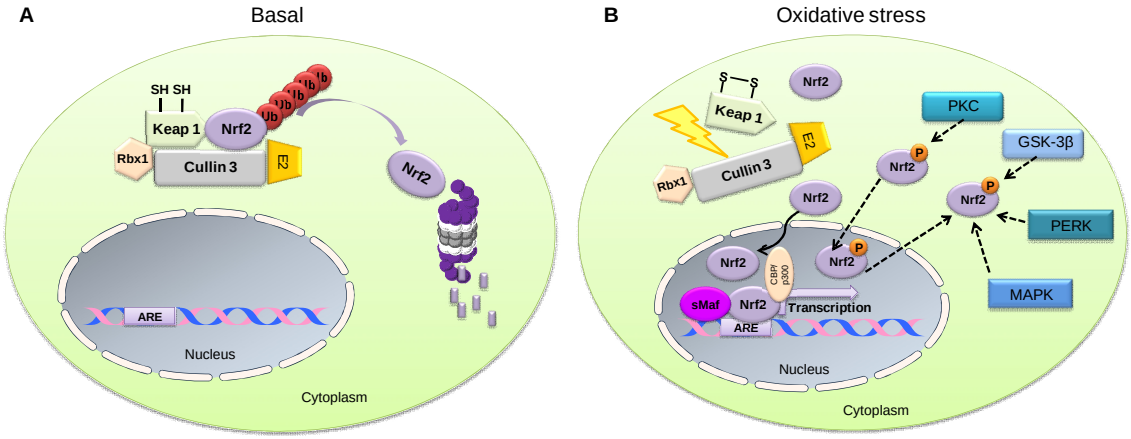

Figure 1.3 Nrf2 main regulatory mechanism. According to the most commonly accepted model of regulation of Nrf2 activity under basal conditions (**A**) Nrf2 is sequestered in the cytoplasm by Keap1, which in association with the Cullin 3 and Rbx1 forms an E3 ubiquitin ligase complex that targets Nrf2 for ubiquitination and proteasomal degradation. Keap1 represses Nrf2 transactivation activity and accelerates its degradation through the proteasome, maintaining low basal expression of Nrf2-regulated genes. Oxidative and electrophilic challenges (**B**) disrupt the complex by modification of reactive cysteine residues of Keap1 thereby leading to Nrf2 stabilization and allowing it to escape proteasomal degradation and translocate to the nucleus. Once in the nucleus Nrf2 heterodimerizes with transcriptional partners, such as small Mafs (sMafs) and CBP/p300, binds to the ARE and recruits the general transcription machinery for expression of ARE-regulated genes. Additionally, in response to oxidative stress and electrophilic compounds, Nrf2 could also be regulated by post-translational modifications, namely phosphorylation by PKC, MAPK, PERK and GSK-3β.

Additionally, in response to oxidative stress and electrophilic compounds, Nrf2 could also be regulated by post-translational modifications. In line with this a number of protein kinases have been implicated in Nrf2 regulation, including protein kinase C (PKC) (Huang et al. 2002) and mitogen activated protein kinase (MAPK) (Yu et al. 1999; Alam et al. 2000). Moreover, glycogen synthase kinase 3β (GSK-3β) has been shown to phosphorylate Fyn which in turn phosphorylates Nrf2 leading to its nuclear export and

thus returning Nrf2 activity to its basal levels (Jain and Jaiswal 2007). Nrf2 has also been shown to be phosphorylated by GSK-3β, PERK (protein kinase R-like endoplasmic reticulum kinase) and CK2 (casein kinase 2), suggesting that phosphorylation could also play an important role in the control of Nrf2 activity (Cullinan and Diehl 2004; Salazar et al. 2006; Pi et al. 2007).

Finally, it was recently reported an alternative mechanism of Nrf2 regulation, independent of Keap1. In these studies the authors suggest that GSK-3β and the E3 ubiquitin ligase β-TRCP regulate Nrf2 through the Neh6 degron in a Keap1-independent manner (Rada et al. 2011; Rada et al. 2012).

1.2.1.2 Nrf2: target genes and biological function

Nrf2 controls the expression of a battery of proteins with antioxidant and anti-inflammatory properties. The orchestrated induction of these proteins, referred to as Nrf2-mediated defense response, is crucial for cells to counteract oxidative stress and maintain cellular redox homeostasis. Data from micro-array analysis have shown that, besides phase II detoxification enzymes and antioxidants, Nrf2 targets include a multitude of genes that can be classified into several categories according to their functions: cellular redox homeostasis, cell growth and apoptosis, inflammatory response, and the ubiquitin-proteasome system (Thimmulappa et al. 2002; Kwak et al. 2003; Rangasamy et al. 2004).

Therefore, Nrf2 regulates the expression of a vast array of functionally diverse proteins that provide cellular protection including proteins involved in the metabolism of xenobiotics, such as NQO1, that catalizes the reduction of quinones and limits the production of ROS and the depletion of intracellular sulfhydryl pools, GST, which catalize the detoxification of endogenous and exogenous compounds through conjugation with glutathione (see section 1.2.2 of this chapter) and multidrug resistance-associated proteins (MRPs), which are responsible for the export out of cells of the conjugation products of several detoxification enzymes, including GST; proteins with antioxidant functions, such as γ-glutamilcysteine ligase (γ-GCL), the rate limiting enzyme in the synthesis of glutathione, χ-CT, the core subunit of the cystine/glutamate membrane transporter, responsible for the uptake of amino acids used in the synthesis of glutathione, HO-1 which contributes to the generation of the antioxidants carbon monoxide and bilirubin (Ryter and Choi 2010) and also the antioxidant proteins thioredoxin,

peroxiredoxin and sulfiredoxin (Ishii et al. 2000; Kensler et al. 2007; Singh et al. 2009). Additionally, Nrf2 also controls the expression of enzymes that promote the synthesis of reducing equivalents (e.g. NADPH), such as glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, and enzymes known to inhibit inflammation, such as leukotriene B4 dehydrogenase. Proteins that are not enzymes but are also involved in cytoprotection, such as ferritin, which protects against iron overload and metallothioneins as well as proteins that participate in the repair and removal of damaged proteins, such as subunits of the 26S proteasome are also targets of Nrf2. In fact, in a recent study, using high-throughput chromatin immunoprecipitation with parallel sequencing methodology more than 600 Nrf2 target genes were identified further confirming the critical role of Nrf2 as a regulator of cell protective and survival responses (Malhotra et al. 2010).

Given the importance of the biological processes in which Nrf2 targets are involved it is not surprising that dysfunction of the Nrf2 pathway can be linked to the pathogenesis of many human diseases. In fact, despite Nrf2 has been originally identified by its role in cancer prevention, evidences have accumulated indicating that it can be protective against several pathological conditions including neurodegenerative diseases, macular degeneration, cardiovascular diseases, ischemia, acute pulmonary injury, asthma, emphysema, pulmonary fibrosis and inflammation (Gao and Talalay 2004; Rangasamy et al. 2004; Kensler et al. 2007; Jazwa and Cuadrado 2010; van Horssen et al. 2010; Baird and Dinkova-Kostova 2011; Jazwa et al. 2011).

As previously referred, oxidative stress has been implicated in the etiology of PD as well as in the MPTP animal model of PD (Jenner 2003; Burton et al. 2006; Chen et al. 2009). DA neurons are particularly vulnerable to oxidative stress due to the highly oxidative environment that surrounds them caused by high levels of iron and dopamine oxidation products (Baez et al. 1997; Jenner 2003). Despite innumerous studies on the role of oxidative stress in PD, data concerning the functional status of the Nrf2-ARE pathway in PD are scarce. Interestingly, activation of the Nrf2-ARE system in PD is suggested by studies demonstrating strong upregulation of nigral immunoreactivity for ARE-regulated proteins, like NQO1 (van Muiswinkel et al. 2004) and HO-1 (Riedl et al. 1999; Schipper 2004), in *postmortem* brain sections. However, this increased expression of ARE-regulated proteins seems largely restricted to (astro)glial cells and is almost absent in dopaminergic neurons. In contrast, investigation of the localization of Nrf2 in the SN of PD brain demonstrated that, in addition to the cytoplasm, a strong nuclear

immunoreactivity is observed in neurons (Ramsey et al. 2007). Interestingly, several studies have demonstrated that Nrf2 expressing astrocytes are able to protect neurons against oxidative stress-induced injury (Shih et al. 2003; Chen et al. 2009; Williamson et al. 2012). Furthermore, Nrf2 ko mice display increased sensitivity to MPTP and overexpression of Nrf2 in glial cells appears to be protective against MPTP-induced toxicity (Chen et al. 2009).

Curiously, it is interesting to note that DJ-1 was demonstrated to stabilize Nrf2 by preventing its association with Keap1 and its subsequent ubiquitination (Clements et al. 2006). Mutations in DJ-1 have been described as cause of some forms of autossomal recessive inherited early onset PD (Bonifati et al. 2003a; Bonifati et al. 2003b). Moreover, in experimental models of PD, DJ-1 was shown to protect against 6-hydroxydopamine and to prevent mutant α-synuclein toxicity, in part by upregulation of glutathione synthesis (Zhou and Freed 2005). As previously referred, the stabilization of Nrf2 may represent an important mechanism in the activation of ARE-dependent gene expression in response to oxidative stress, which include the genes encoding for proteins involved in glutathione synthesis. Without intact DJ-1, the Nrf2 protein becomes unstable, compromising Nrf2-induced transcriptional activity both under basal conditions and after exposure to inducing agents like ROS (Clements et al. 2006).

1.2.2 Glutathione S-transferases

Glutathione S-transferases (GST; EC 2.5.1.18) are phase II drug metabolizing enzymes belonging to a multigene family of isoenzymes whose primary function is to catalyze the detoxification of endogenous and exogenous compounds through conjugation of the nucleophilic thiol-reduced glutathione (GSH) to an electrophilic carbon, sulphur or nitrogen atom on these compounds, generally rendering the resultant conjugates more soluble and thus easily eliminated from the cell (Mannervik and Danielson 1988; Hayes and Pulford 1995; Hayes et al. 2005). GST are therefore involved in the detoxification of both electrophilic xenobiotics, such as chemical carcinogens, pesticides and other environmental pollutants, and antitumor agents; and secondary products of the endogenous metabolism, such as hydroperoxides and other ROS, resultant from OXPHOS, and quinones formed, for instance, as a result of dopaminergic metabolism (Baez et al. 1997; Hayes et al. 2005).

Besides their critical role in detoxification of cells from several toxic chemicals and products of oxidative stress, GST possess important non-catalytic properties, some that have been previously described, as specific binding and transport of hormones and bile acids (Litwack et al. 1971; Hayes and Pulford 1995; Hayes et al. 2005), and some that have been highlighted in the past years, namely as intervenients in post-translational modifications, such as *S*-glutathionylation, and regulators of intracellular signaling pathways, through direct protein-protein interactions (Tew 2007; Tew and Townsend 2011).

In mammals, three main families of GST exist, that can be divided in soluble GST, comprising cytosolic and mitochondrial/peroxisomal GST, and microsomal GST also referred to as membrane-associated proteins in eicosanoid and glutathione metabolism (MAPEG) (Hayes et al. 2005; Higgins and Hayes 2011; Tew and Townsend 2012). Cytosolic GST are grouped in seven different classes[1], alpha (α, A), mu (μ, M), pi (π, P), omega (ω, O), sigma (σ, S), theta (θ, T) and zeta (ζ, Z), classified according to the percentage of shared homology, with special focus on the highly conserved catalytic residues at the N-terminal: tyrosine, serine or cysteine residues (Hayes et al. 2005; Mannervik et al. 2005; Higgins and Hayes 2011; Tew and Townsend 2012). Cytosolic GST are catalytically active in the form of homo- or heterodimers with the dimer interface providing a non catalytic site for ligand binding (Mannervik and Danielson 1988; Pettigrew and Colman 2001; Hayes et al. 2005; Gildenhuys et al. 2010). GST isoenzymes possess a substrate binding site (H-site) and a GSH binding site (G-site), through which the residue in the active catalytic site interacts with the thiol group of GSH (Dirr et al. 1994). A common feature of the various GST isoenzymes, which accounts for their catalytic activity, is that they possess the ability to lower the pK_a of the thiol group of GSH, allowing the formation of thiolate anion (GS^-), at physiological conditions (Higgins and Hayes 2011).

GST potential roles in drug metabolism and chemical toxicity, tumor etiology and development of anticancer drug resistance, degenerative and inflammatory disorders, and in providing protection against oxidative stress have attracted great research interest in this area (Hayes et al. 2005; Henderson and Wolf 2011; Tew and Townsend 2012).

[1] Previous nomenclature used the Greek alphabet, newer nomenclature uses Latin script. Genes are thus designated for example *GSTA1* (or *Gsta1* in the mouse), with the corresponding homodimeric protein being referred to as GSTA1-1, with the prefix added to distinguish GST from different species (e.g. mGSTA1-1, for mouse GST)

GST are ubiquitously expressed polymorphic enzymes that have been described to determine the susceptibility to a diverse array of human diseases, ranging from different types of cancer (Bolt and Thier 2006; Lo and Ali-Osman 2007; Economopoulos and Sergentanis 2010; Josephy 2010; Backos et al. 2012), to asthma (Mapp et al. 2002; Child et al. 2003; Aynacioglu et al. 2004), and from inflammatory diseases (Mattey et al. 1999; Kim et al. 2006a) to PD (Kelada et al. 2003; Golbe et al. 2007; Vilar et al. 2007).

The earliest (and the vast majority of) epidemiological studies on human GST polymorphisms focused in the null genotypes of *GSTM1* and *GSTT1* (i.e., are homozygous for *GSTM1*0* and/or *GSTT1*0* alleles), and the allelic variants of *GSTP1* that encode enzymes with reduced catalytic activity (Bolt and Thier 2006; Lo and Ali-Osman 2007; Josephy 2010). Due to some conflict in the results obtained in these studies, a number of meta-analyses have been carried out as part of the Human Genome Epidemiology Network (HuGE), that provided evidence that GST polymorphisms can result in an increased risk of some types of cancer, albeit relatively small in some cases (Josephy 2010).

Thus, although several types of cancer display increased expression levels of GST proteins, which might be associated to both increased susceptibility to carcinogenesis, and resistance to chemotherapeutic drugs (conferred by some polymorphic variants), a relatively weak effect of these polymorphisms on the risk of cancer was observed (Hayes and Pulford 1995; Hayes et al. 2005). In contrast, several studies indicate that loss of these genes increase susceptibility to asthma and allergies, atherosclerosis, rheumatoid arthritis, and systemic sclerosis (Palmer et al. 2003; Gilliland et al. 2004; Romieu et al. 2004). Additionally, class alpha GST represents quantitatively a major group of transferases in the liver, having substantial influence in detoxification processes and it has been shown that polymorphisms in *GSTA1* and *GSTA2* influence both the amount of synthesized protein and the activity of the encoded proteins (Morel et al. 2002; Ning et al. 2004; Tetlow and Board 2004).

The impact of GST polymorphisms on disease susceptibility and therapeutic outcomes has been extensively studied in the context of its phase II detoxification properties, but the role of these polymorphisms as mediators of *S*-glutathionylation or protein-protein interactions is less well explored (Tew and Townsend 2012). Advances in the understanding of how GST polymorphisms affect the susceptibility to disease as well as the mechanisms of therapeutic efficiency and drug resistance will be crucial to perform

patient selection based on biomarker profiles and to adapt and individualize therapeutic strategies, improving therapeutic outcomes in a multitude of diseases.

The understanding of the physiological roles of GST is a critical important issue that can be addressed through the deletion of the GST gene encoding for each specific GST enzyme and determination of the phenotypic consequences. Targeted disruption of murine genes has demonstrated, for instance, that cytosolic GST isoenzymes are broadly cytoprotective, whereas MAPEG proteins display proinflammatory activities (Hayes et al. 2005). However, in multigene families of enzymes, such as GST, where genetic and functional redundancy is common, it is often difficult to comprehensively define the role of a specific enzyme when other gene products can, at least partially, compensate for the loss of function (Henderson et al. 2003; Henderson and Wolf 2011), as testified by reports on the knockout of mouse *GSTZ1* leading to overexpression of enzymes of the alpha, mu and pi classes (Lim et al. 2004; Blackburn et al. 2006). A further complication is that all gene knockout work has been, traditionally, carried out in the mouse, and different strains have quite different genetic background that may modify, mask or significantly influence, the phenotypic effects of gene deletions (Jiang et al. 2011). Nevertheless, the use of GST ko mice is an instrumental model for the study of several diseases, where GST polymorphisms are relevant, as well as for uncovering new therapeutic targets and strategies. Particularly with the advances in transgenic technology that nowadays allow not only the deletion of a specific gene but also make possible the replacement of that gene for their orthologous human counterpart, making possible the study of the expression and activity of that gene in several disease models (Henderson and Wolf 2011).

1.2.3 Glutathione S-transferase pi (GSTP)

1.2.3.1 GSTP polymorphisms

Human *GSTP* localizes in chromosome 11 and has 2 polymorphic sites in exon 5: at codon 105, where a nucleotide transition from adenosine-to-guanine (A-G) causes an isoleucine-to-valine substitution (Ile105Val); and at codon 114 where occurs an alanine-to-valine substitution (Ala114Val). The polymorphic variants arising from these transitions generate four haplotypes of GSTP: wild-type GSTP1*A (Ile105/Ala114), GSTP1*B (Val105/Ala114), GSTP1*C (Val105/Val114), and GSTP1*D (Ile105/Val114)

(Ali-Osman et al. 1997; Hayes and Strange 2000; Hayes et al. 2005; Vasieva 2011). Although the Ile105Val and Ala114Val substitutions do not affect GSH-binding affinity, they do cause alterations in the substrate-binding site of the enzyme impacting enzymatic activities of GSTP1*B and GSTP1*C alleles (Pandya et al. 2000; Ishimoto and Ali-Osman 2002). The substrate specificity of each GSTP1 isoenzyme might be determined by the size and hydrophobicity of the residue 114 (Hu et al. 1998). In turn the amino acid 105 proximity to GSTP catalytic center can directly influence its catalytic activity (Pandya et al. 2000; Ishimoto and Ali-Osman 2002). Thus GSTP polymorphisms can affect substrate selectivity and stability increasing individual susceptibility (Hayes and Strange 2000).

In fact, individuals with GSTP1*B allele have lower GSTP enzyme activity and thus display less efficient detoxification capacity (Ishimoto and Ali-Osman 2002). Moreover, this polymorphism confers higher susceptibility to several types of disease, including a number of different cancers (Lee et al. 2000; Risch et al. 2001; Economopoulos and Sergentanis 2010), asthma (Mapp et al. 2002; Child et al. 2003; Aynacioglu et al. 2004), inflammatory disorders (Mattey et al. 1999; Kim et al. 2006a) and Parkinson's disease (Kelada et al. 2003; Golbe et al. 2007; Vilar et al. 2007).

As it is the case for other GST, also for GSTP polymorphism controversy data have derived from different studies (Ye et al. 2006; Josephy 2010). The explanation could be the framing in different genetic backgrounds and the induction of compensatory functions that can mask the effects of GSTP mutation (Henderson and Wolf 2011).

Accurate epidemiological information will establish the role of these polymorphisms in the individual responsiveness to therapies, a critical issue on the development of novel therapeutic drugs in order to improve efficiency and reduce both side effects and drug resistance.

1.2.3.2 GSTP functions: detoxification and beyond

The GSTP class of isoenzymes integrates the cytosolic family of GST and is structurally and functionally related with GSTM and GSTA, sharing conservative motifs at sites important for their catalytic and protein-binding functions (Hayes and Pulford 1995; Vasieva 2011). Alike other GST, the most traditionally attributed and well-characterized function of GSTP is its catalytic function of detoxification by conjugating GSH with several endogenous and exogenous electrophilic compounds (Tew

and Townsend 2011; Vasieva 2011; Tew and Townsend 2012). The overlapping substrate specificity within cytosolic GST classes difficult the identification of isoenzymes based only on their catalytic properties (Hayes et al. 2005).

The catalytic detoxification properties of the GSTP class (as well as others GST), have been a primary research focus for the last decades. However, in the last few years novel pathways, that extend beyond the catalytic detoxification function of GSTP, and explain the striking role of GSTP in the susceptibility to several human diseases, have been discovered. The emergence of these novel non-catalytic functions has drawn attention to the biological importance of this particular class of GST. GSTP has been shown to protect cells from ROS by modulating *S*-glutathionylation of proteins, following oxidative and nitrosative stress (Klatt and Lamas 2000; Manevich et al. 2004; Tew 2007; Townsend et al. 2009a), altering the levels of glutathione (Baez et al. 1997; Tew and Ronai 1999), and also by acting as a ligand-binding protein controlling the catalytic activity of kinases involved in cell-signaling pathways (Adler et al. 1999; Wang et al. 2001; Wu et al. 2006).

1.2.3.3 Regulatory functions of GSTP: modulation of signaling pathways

A recently described non-catalytic function of GSTP that can impact cell death and survival is the ability to establish protein-protein interactions with kinases involved in cell-signaling pathways. Interestingly it has been demonstrated that GSTP directly targets at least three proteins in the same stress-responsive pathway: tumor necrosis factor alpha (TNF-α)-receptor associated factor 2 (TRAF2) and its downstream targets, JNK and apoptosis signal-regulating kinase 1 (ASK1) (Figure 1.4) (Adler et al. 1999; Yin et al. 2000; Wang et al. 2001; Elsby et al. 2003; Wu et al. 2006).

JNK is a member of the MAPK family, that regulates a multitude of physiological and pathological processes and is involved in diseases as disparate as cancer and neurodegenerative disorders (Davis 2000; Davies and Tournier 2012). The JNK cascade, also referred to as stress-activated signaling pathway, mediates the activation of the apoptotic cell response to ROS, heat-shock and pro-inflammatory cytokines, (Davis 2000; Davies and Tournier 2012) and is thus, transiently upregulated by these signals. However, JNK activation is also associated with the regulation of cell survival, proliferation and differentiation (Davis 2000). The apparently antagonistic functions of JNK in the

modulation of these cellular pathways might depend on signal intensity and duration to discriminate which pathway is to be activated. Accordingly, evidences suggest that transient JNK activation promotes cell survival, whereas prolonged JNK activation induces cellular apoptosis (Ventura et al. 2006).

JNK is activated by dual phosphorylation, mediated by the MAPK kinases (MAPKK or MKK) MKK4 and MKK7, on a Thr-Pro-Tyr motif located within the subdomain of the activation loop in the protein kinase (Davis 2000; Davies and Tournier 2012). In turn MKK4 and MKK7 are activated by phosphorylation on serine and threonine residues by an upstream kinase (MAPKKK or MKKK) (Davis 2000; Davies and Tournier 2012). MKK4 and MKK7 function in a non-redundant, synergistic manner, and have distinct affinities for JNK, with MKK4 preferentially phosphorylating JNK on tyrosine and MKK7 preferentially phosphorylating JNK on threonine residues respectively (Lin et al. 1995; Haeusgen et al. 2011; Davies and Tournier 2012). The downstream events activated by this signaling cascade involve the JNK mediated phosphorylation of several transcription factors, namely c-Jun, activating transcription factor 2 (ATF2) and p53, as well as anti- and pro-apoptotic members of the Bcl-2 family, contributing to the cellular stress response through alterations in the cell cycle, DNA repair and apoptosis (Davis 2000; Tew and Townsend 2012).

GSTP was demonstrated to bind to the C-terminus of JNK, through direct protein-protein interaction, acting as an endogenous negative regulator and maintaining low basal levels of its kinase activity (Figure 1.4) (Adler et al. 1999; Wang et al. 2001). Both the C-terminal domain and the active center region of GSTP are required for regulation of the protein-protein interaction (Adler et al. 1999; Monaco et al. 1999; Wang et al. 2001; Asakura et al. 2007). However, the catalytic activity of GSTP is not affected by protein binding, which implies that the ligand-binding effects are mediated by different regions than the ones involved in GSH or substrate binding (Tew and Townsend 2012).

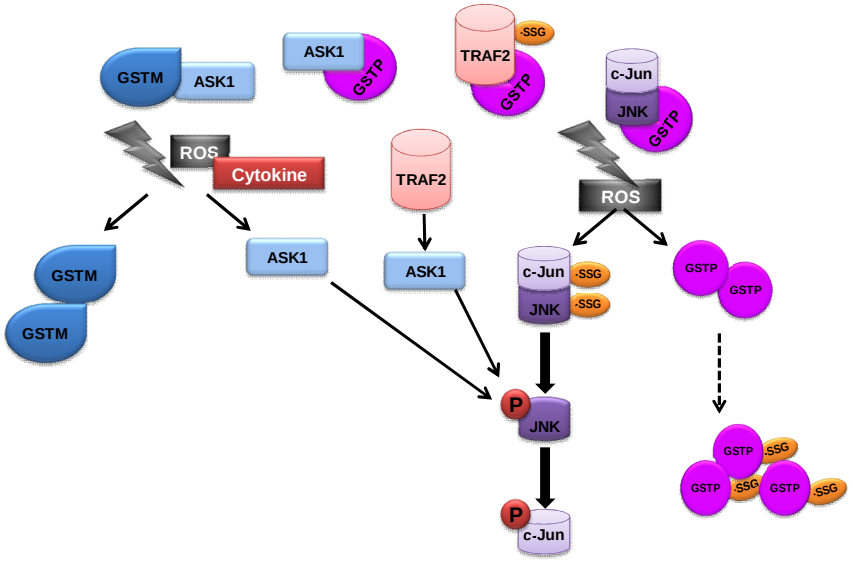

Figure 1.4 Protein phosphorylation and S-glutathionylation in cell signalling: GST as ligand binding proteins of cellular kinases. Protein:protein interactions between GSTM or GSTP and regulatory kinases are depicted. In non-stressed cells, low JNK activity is maintained through direct interaction and repression of JNK activity by GSTP. Following exposure to reactive oxygen species (ROS), GSTP dissociates from the complex, accumulating as GSTP oligomers with resultant activation of released JNK (indicated by -P) that may activate its downstream targets, namely c-Jun. Additionally, under oxidative stress, GSTP associate with the target proteins c-Jun, JNK and TRAF2, which are modified by *S*-glutathionylation (as indicated by –SSG) altering their activity. Moreover, GSTP can also enable its own *S*-glutathionylation. In a similar mechanism GSTM associates with ASK1 and upon different stimuli (e.g. ROS, cytokines) the complex is disrupted and ASK1 is activated and activates its downstream targets that include JNK. Dashed line indicate hypothetical, rather than experimental established, steps. *Adapted from Tew and Townsend 2012* and *Townsend 2007*.

Upon oxidative stress conditions GSTP dissociates from the GSTP-JNK complex with resultant activation of JNK and its downstream targets, and consequent activation of cellular stress response, apoptosis and proliferation (Yin et al. 2000). The allosteric inhibition of GSTP is accompanied of a conformational change that can also enables the release and activation of JNK, triggering the apoptotic process through the mitochondrial pathway (Aoki et al. 2002; Asakura et al. 2007). Therefore, through the modulation of JNK activity, GSTP may serve as an endogenous regulator of the cellular stress response, eliciting protection against cell death induced by ROS (Adler et al. 1999; Yin et al. 2000).

GSTP is mainly expressed in the cytoplasm, however it was also reported its nuclear localization in cancer cells, where it was shown to prevent damage to the DNA by acting as a scavenger of lipid peroxides (Kamada et al. 2004). Some studies have also demonstrated that GSTP can be localized at the mitochondria (Gallagher et al. 2006; Goto et al. 2009; Raza 2011). The mitochondrial putative localization of GSTP could be

relevant to both the control of ROS generated upon mitochondrial dysfunction, and to constrain the mitochondrial pathway of apoptosis triggered by JNK signaling.

Interestingly, GSTA, which has structural and functional homology with GSTP, is able to suppress JNK signaling caused by inflammatory cytokines or oxidative stress by a similar mechanism (Romero et al. 2006).

Elevated levels of GSTP in drug-resistant tumor cells have long been reported, even when the drug is not a substrate for GSTP (Tew 1994). This fact could now, in the light of the GSTP binding function, be explained by the need to restrain JNK levels in tumors where the JNK-induced apoptosis is suppressed (Tew 2007).

Evidences suggest that JNK plays a role in neurodegeneration in sporadic PD (Peng and Andersen 2003; Hunot et al. 2004) and it has also been shown that JNK is involved in MPTP-induced neuronal damage in experimental models of PD (Nishi 1997; Saporito et al. 2000; Gearan et al. 2001; Kuan and Burke 2005). Additionally, JNK ko mice display increased resistance to MPTP-induced DA cell death (Hunot et al. 2004). Conversely, GSTP ko mice present increased constitutive JNK activity in the liver and lung (Elsby et al. 2003). Recently by proteomic approach GSTP has also been identified as being involved in PD progression (Shi et al. 2009).

GSTP has also been reported to associate with TRAF2, thus inhibiting both JNK and p38, but not NF-κB (Wu et al. 2006). This mechanism allows TNF-α signalling regulation by GSTP and provides an additional mechanism for the regulation of cell death pathways in both ways: through the inhibition of TRAF2-ASK1-induced apoptosis, since it suppresses the interaction between these two proteins; and as an additional form of regulation upstream of the JNK signalling, given that ASK1 is a MKKK that activates both p38 and JNK pathways (Figure 1.4). This can therefore be a mechanism of regulation upstream of the cytokine- and stress-induced apoptosis (Ichijo et al. 1997). GSTP can, therefore, provide a broad control of the entire pathway from the receptors to the transcription factors, putting it in a privileged position for therapeutic targeting.

GSTM1, another cytosolic GST with homology to GSTP, can also bind to and inhibit ASK1 (Figure 1.4) (Cho et al. 2001; Dorion et al. 2002), by a mechanism analogous to the GSTP-JNK binding, providing another example of functional redundancy within the GST family.

1.2.3.4 Regulatory functions of GSTP: role on S-glutathionylation

S-glutathionylation is a post-translational modification which consists in the conjugation of glutathione to low-pK_a cysteine sulfhydryl or sulfenic acid moieties in target proteins, through a disulfide bond (Figure 1.5). Cysteine residues in the vicinity of Lys, Arg or His residues, by virtue of their basic environment, are more susceptible to *S*-glutathionylation. The addition of glutathione introduces a negative charge onto the protein (as a consequence of the insertion of glutamic acid), and increases its molecular weight by ~305 Da, which likely alters protein conformation (Townsend 2007; Tew and Townsend 2011).

Although it also occurs under physiological conditions, *S*-glutathionylation is favored in conditions of oxidative and/or nitrosative stress. *S*-glutathionylation is a reversible process of protein regulation that protects proteins from further (and possibly irreversible) oxidation, and could alter both function and cellular localization of the proteins (Townsend 2007; Tew and Townsend 2011).

A critical factor for considering *S*-glutathionylation as a regulatory post-translational modification, similar to the well-known process of phosphorylation/dephosphorylation, is the reversibility of the process. In fact, several reports have established a role for cysteine-rich proteins, such as thioredoxin, sulfiredoxin and glutaredoxin (Grx) in the reversal glutathionylation process (deglutathionylation) (Shelton et al. 2005; Findlay et al. 2006; Tew and Townsend 2011). Moreover, Grx was identified as being involved in the thiol-disulfide exchange reaction, meaning that it catalizes both the forward and reverse reactions of the *S*-glutathionylation (Gravina and Mieyal 1993; Tew and Townsend 2011; Sabens Liedhegner et al. 2012). Another protein that has been implicated as a putative deglutathionylation enzyme is protein disulfide isomerase (PDI) (Nakamura et al. 1996). PDI is localized to the endoplasmic reticulum and is involved in protein folding through disulfide bond formation and isomerization. Reversibility of the *S*-glutathionylation reaction, either spontaneously, by glutathione, or catalytically, by glutaredoxin or sulfiredoxin, provides the cell with a dynamic cycle of regulatory events – the *S*-glutahionylation cycle.

Interestingly, results from Tew and collaborators have shown that GSTP potentiates *S*-glutathionylation reactions in response to oxidative and nitrosative stress (Figure 1.5) (Townsend et al. 2009a). Catalysis of this post-translational modification is a

novel property for GSTP and an important finding in the understanding of the *S*-glutathionylation cycle, namely in respect to the forward reaction, since it is one of the few proteins that has been demonstrated to catalyze the forward reaction of *S*-glutathionylation (Xiong et al. 2011).

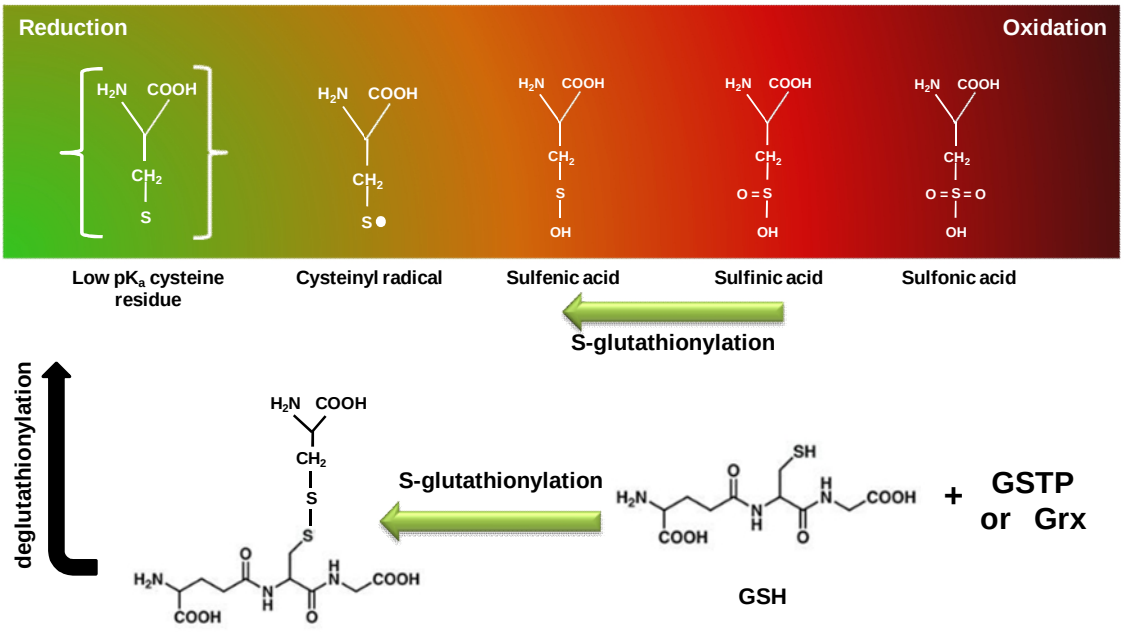

Figure 1.5 Schematic representation of cysteines oxidation and reduction processes, including *S*-glutathionylation. Oxidation states of sulfur following exposure to electrophilic oxygen or nitrogen species determine the intermediates of cysteine modifications in proteins. Whereas the "sulfonic acid" state generally drives the protein bearing that modified cysteine to degradation, "sulfenic acid" and "sulfinic acid" residues can generally be rescued/reduced through *S*-glutathionylation. The forward *S*-glutathionylation reaction of the "*S*-glutathionylation cycle" can be facilitated by GSTP or glutaredoxin (Grx), with GSH or GSSG as proximal donors. The reverse reaction – "deglutathionylation" can be effected by glutaredoxin, sulfiredoxin or thioredoxin. *Adapted from Tew 2007*.

In fact, Fisher and collaborators have previously demonstrated an important role for GSTP in catalyzing the conjugation of glutathione with 1-Cys peroxiredoxin (1-CysPrx), by heterodimerization, resulting in its activation (Manevich et al. 2004).

1-CysPrx belongs to the peroxiredoxin superfamily and is expressed in all major organs, with particularly high levels in brain, eye, testes, and lung (Singh and Shichi 1998; Rhee et al. 2001; Manevich et al. 2004). 1-CysPrx is a dual-function enzyme with both GSH-peroxidase (GPx) and acidic calcium-independent/lysosomal phospholipase A2 activities that protects cells against membrane oxidation through GSH-dependent reduction of phospholipid hydroperoxides to corresponding alcohols (Kang et al. 1998;

Rhee et al. 2007; Tew and Townsend 2011). However, purified native or recombinant enzyme *in vitro* generally lacks GPx activity due to the oxidation of its single conserved cysteine to sulfinic acids (Kang et al. 1998; Rhee et al. 2007). By virtue of the 1-CysPrx structure, the reduction of its oxidized cysteine is difficult, because it is not accessible by thioredoxin or sulfiredoxin. Reversible oxidative (sulfenic acid) inactivation of this enzyme is similar to other peroxiredoxins, but it is also not accessible to GSH.

Importantly, Fisher and collaborators have shown that heterodimerization of 1-cysPrx with GSH-saturated-GSTP results in *S*-glutathionylation of the oxidized cysteine in 1-cysPrx followed by subsequent GSH-dependent reduction of the mixed disulfide and restoration of enzymatic activity. Thus, reactivation of 1-CysPrx by GSTP overcomes an accessibility barrier for GSH to regenerate sulfhydryls (Manevich et al. 2004). 1-CysPrx is an important player in the antioxidant defense and it was found in high levels in the brain of PD patients (Power et al. 2002). Moreover, it was also shown that JNK and 1-CysPrx compete for GSTP and affect activation of each other (Kim et al. 2006b).

This GSTP non-catalytic function has also been identified in the ischemic heart, where aldose reductase (AR) is a target for the forward catalysis of *S*-glutathionylation by GSTP (Wetzelberger et al. 2010). AR is a multifunctional enzyme that catalyzes the reduction of glucose and lipid peroxidation-derived aldehydes. During myocardial ischemia, the activity of AR is increased due to the oxidation of its cysteine residues to sulfenic acids. The conversion of the activated, sulfenic form of the protein (AR-SOH), back to its reduced inactivated state (AR-SH), comprises an intermediary state during reperfusion, when AR-SOH is converted to a mixed disulfide (AR-SSG) that is then reduced to AR-SH by Grx. The conversion to inactive state of AR is delayed in GSTP ko mice. Thus, in ischemic hearts, AR co-immunoprecipitates with GSTP, whereas in reperfused hearts, the association of AR with Grx is increased (Wetzelberger et al. 2010). These findings suggest that the sequential catalysis by GSTP and Grx might be a general redox switch mechanism that regulates the reduction of protein sulfenic acids to cysteines (Figure 1.5).

Additionally, GSTP is itself regulated by *S*-glutathionylation on Cys47 and Cys101 residues, and this modification leads to a reduction in the catalytic activity of GSTP and to a disruption of the GSTP-JNK complex, providing a self-regulatory mechanism for GSTP (Townsend et al. 2009a). In fact, within this family of small redox

active proteins, besides GSTP, also Grx (Fratelli et al. 2002), and PDI (Townsend et al. 2009b) are regulated by *S*-glutathionylation.

The *S*-glutathionylation cycle has the potential to selectively regulate the function of a number of enzymes, receptors structural proteins, transcription factors and transport proteins and may alter a variety of protein-protein interactions. Proteins so far identified as susceptible to *S*-glutathionylation can be grouped in six different clusters, accordingly to the cellular processes or pathways in which they are involved: 1) Cytoskeletal constituents; 2) Energy metabolism/Glycolysis and Mitochondrial; 3) Signaling proteins (particularly kinases and phosphatases); 4) Calcium Homeostasis; 5) Protein Folding; 6) Redox regulation (Townsend 2007; Tew and Townsend 2011). Alike phosphorylation and other important post-translational modifications, *S*-glutathionylation, and cysteine modification in general, is critical to cellular signaling and its deregulation could have impact in several human diseases. This process is being increasingly recognized as an important biological mechanism involved in the homeostatic redox control (Townsend 2007; Xiong et al. 2011).

1.3 Ubiquitin-Proteasome System (UPS)

The dynamic state of intracellular proteins is maintained in equilibrium by the processes of protein synthesis and protein degradation (Glickman and Ciechanover 2002; Schwartz and Ciechanover 2009). The UPS is the primary proteolytic complex responsible for the degradation of short-lived proteins and provides the specificity and temporal control needed for fine-tuning the steady-state levels of many regulatory proteins (Glickman and Ciechanover 2002; Ciechanover and Brundin 2003; Schwartz and Ciechanover 2009). Therefore, in addition to mediating the degradation of damaged and misfolded intracellular proteins, through the regulation of protein turnover, the UPS also regulates the function of several proteins, including transcription factors, many of which are critical in the determination of cell fate (Thompson et al. 2008; Bhat and Greer 2011). The UPS has in fact been demonstrated to play crucial roles in numerous cellular functions including regulation of cell cycle and division, DNA damage repair, cellular stress response, signal transduction, membrane trafficking, neural development and transcription (Glickman and Ciechanover 2002; Ciechanover and Brundin 2003; Schwartz and Ciechanover 2009; Bhat and Greer 2011).

The degradation of proteins by the UPS is a sequential process involving an initial step of ubiquitin conjugation to the protein substrate followed by the degradation of the tagged protein through the 26S proteasome complex, with release of free ubiquitin, mediated by deubiquitinating enzymes (DUBs) (Glickman and Ciechanover 2002; Ciechanover and Brundin 2003; Schwartz and Ciechanover 2009; Bhat and Greer 2011).

1.3.1 Ubiquitin and Ubiquitination machinery

Ubiquitin (Ub) is a highly conserved 76 amino acid protein, which localizes in the cytosol and nucleus, and is ubiquitously expressed in eukaryotic cells (Goldstein et al. 1975; Ciehanover et al. 1978; Hershko and Ciechanover 1998). Ub is encoded by a multigene family and expressed as three mainly precursors: a head-to-tail polymer of identical subunit repeats (polyubiquitin) and two ubiquitin moieties fused to the ribosomal polypeptides L40 and S27. Upon translation of the Ub RNA, these precursor molecules are cleaved by specific endopeptidases to yield identical free Ub monomers (Jentsch et al. 1991; Glickman and Ciechanover 2002; Catic and Ploegh 2005).

Ub conjugation, or ubiquitination, is a post-translational modification in which Ub is linked to substrate proteins through isopeptide bonds between the C-terminal glycine residue of Ub and the ε-amino group of lysine residues in the proteins. Ubiquitination consists in the covalent attachment of one (monoubiquitination) or several (polyubiquitination) Ub molecules to a protein and depends on the concerted, successive action of three types of enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzymes (E2) and the ubiquitin protein ligases (E3) (Hershko and Ciechanover 1998; Glickman and Ciechanover 2002; Schwartz and Ciechanover 2009). Whereas the attachment of multiple Ub molecules is generally associated with the degradation of the Ub-tagged protein substrate by the proteasome, monoubiquitination can serve different functions and result in protein translocation, internalization and modification of function (Thompson et al. 2008; Bhat and Greer 2011). In fact monoubiquitination has been linked, for instance, to the regulation of transcription, through the regulation of histones and transcription factors (Muratani and Tansey 2003; Bhat and Greer 2011).

Ub conjugation to protein substrates proceeds via a three step cascade mechanism (Figure 1.6): initially the E1 enzyme activates Ub, in an ATP-dependent reaction, which involves the formation of an ubiquitin-adenylate intermediate and the transfer of this

intermediate to a specific cysteine in the active site of the E1 enzyme through a high-energy thiol ester bond formed between the C-terminal glycine residue of Ub and the E1 cysteine residue (Haas and Rose 1982; Haas et al. 1982; Glickman and Ciechanover 2002). Subsequently, one of several E2s transfers the activated Ub from E1 *via* another high-energy thiol ester intermediate (E2~Ub) to the substrate (Pickart and Rose 1985; Haas and Bright 1988). E2 enzymes catalyze the covalent attachment of Ub to a lysine residue in the substrate proteins, and can act in concert with E3 enzymes (Hershko et al. 1983).

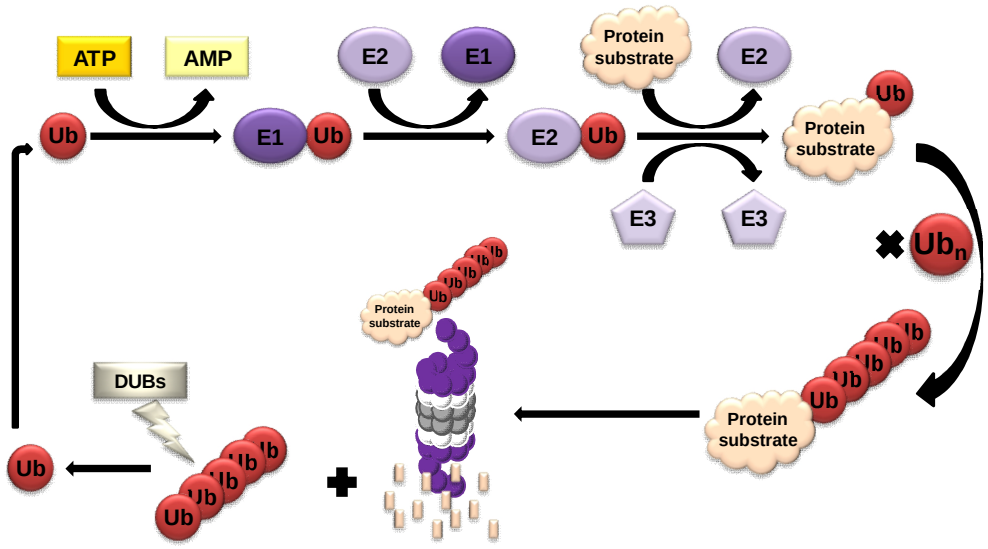

Figure 1.6 Ubiquitination and proteasome degradation. Ubiquitin (Ub) conjugation to protein substrates proceeds via a three step cascade mechanism. In the first step the ubiquitin-activating enzyme (E1) activates Ub, through the formation of a high-energy thiol ester bond between the C-terminal glycine residue of Ub and a specific cysteine in the active site of the E1 enzyme, in an ATP-dependent reaction. Subsequently, Ub is transferred to one of several ubiquitin-conjugating enzymes (E2) to which Ub is also linked *via* a thiol ester bond. Some E2 enzymes catalyze the covalent attachment of Ub to a lysine residue in the substrate proteins directly, whereas other E2 enzymes act in concert with ubiquitin protein ligases (E3). The consecutive addition of activated Ub moieties to internal lysine residues on the previously conjugated Ub molecule leads to the formation of a polyubiquitin chain. The ubiquitinated proteins are then recognized and degraded by the 26S proteasome complex whereas Ub is released, by the action of deubiquitinating enzymes (DUBs), and can thus be reused.

Different classes of E3 exist, the two major groups being: the HECT (homologous to the E6-AP COOH terminus) domain E3s and the RING (really interesting new gene) finger-containing E3s. The mechanism of action of HECT E3s involves the transference of Ub from the E2 to a cysteine in the active site of the E3, with the formation of another thiol ester intermediate (E3-Ub), before it is finally transferred to the ligase-bound substrate. On the other hand, RING finger containing E3s catalize the direct transfer of

the activated moiety from E2 to the E3-bound substrate. Thus, the combination of E2s and E3s determines the specific recognition of substrates. The consecutive addition of activated Ub moieties to internal lysine residues on the previously conjugated Ub molecule leads to the formation of a polyubiquitin chain. The ubiquitinated proteins can then be recognized and degraded by the 26S proteasome complex whereas Ub is released, by the action of DUBs, and can thus be reused (Hershko and Ciechanover 1998; Glickman and Ciechanover 2002).

1.3.2 Proteasome

Proteins with a chain of more than four Ub molecules attached are targeted for degradation by the proteasome (Thrower et al. 2000). The 26S proteasome is a large multimeric protease complex with more than 30 different subunits and approximately 2.5 MDa. The 26S proteasome is formed by a 20S core particle (CP), which contains the protease subunits, capped on one or both ends by the 19S regulatory particles (RP), that regulates the proteolytic function of the protease core (Glickman and Ciechanover 2002; Kim et al. 2011).

The 20S CP is a barrel-shaped structure composed of 28 subunits, forming four heptameric rings. The two inner β-rings contain the proteolytic active sites facing inward into the proteolytic chamber. The α-subunits in the outer rings of the 20S CP can recognize and direct polyubiquitinated substrates into the proteolytic chamber. One or two 19S RP can be attached to the surface of the outer α-rings of the 20S CP to form the 26S proteasome holoenzyme (Kim et al. 2011).

The 19S RP is a 700 KDa complex also called proteasome activator 700 or PA700, and is formed by two substructures, a lid and a base, with multiple subunits. The 19S RP possesses six homologous ATPases (Rpt1-6) and at least three non-ATPases subunits (Rpn1, Rpn2 and Rpn10) in the base and more than ten non-ATPases subunits in the lid. The association between the base and the lid is mostly mediated by Rpn10. Direct interactions have been reported between the six ATPases in the base and the α-subunits of the 20S CP. The ATPases in the base most likely recognize, unfold and translocate substrates through a gated channel into the 20S CP. Degradation of ubiquitinated substrates requires the 19S RP lid and the previous deubiquitination by DUBs present in the lid (Young et al. 1998; Yao and Cohen 2002; Kim et al. 2011).

OBJECTIVES

The research studies presented in this thesis are mainly focused on the characterization of the putative neuronal protective role of GSTP against oxidative stress and proteasome inhibition. Besides its well characterized role in detoxification of endogenous and exogenous potential harming electrophilic compounds, GSTP has recently been described to have other non-catalytic functions in the modulation of important cell-death and survival pathways. These new functions of GSTP have not yet been thoroughly explored.

Therefore, the main goals of this work were: to provide novel insights into the pathways involved in dopaminergic neurodegeneration in an MPTP mouse model of PD and to elucidate the molecular mechanisms through which GSTP might regulate this pathways to achieve neuronal protection. Additionally, we also aimed to investigate the effect of MPTP-induced oxidative stress in UPS function and to evaluate the additional contribution of the impairment of this pathway to the neurodegenerative process, as well as assess the mechanisms of action of GSTP in this context.

The experimental approaches used involved a wide range of biochemical and molecular biology techniques and were designed to address the following specific questions:

1. What is the impact of the absence of GSTP (GSTP knockout mice) on the response to MPTP-induced oxidative stress?
2. Does GSTP interact with JNK signaling pathway *in vivo*?
3. Is the Keap1-Nrf2 protein-protein interaction regulated by GSTP-induced *S*-glutathionylation *in vivo*?
4. Is GSTP expression regulated by a feedback positive loop involving increased expression of Nrf2?
5. Is the constitutive activity of the UPS altered in GSTP knockout mice?

6. Is the UPS activity differentially modulated in wild-type *vs* GSTP knockout mice exposed to the neurotoxin MPTP or proteasome inhibition?

Taken together, the results presented herein provide a significant contribution to the understanding of the molecular mechanisms underlying GSTP-elicited neuronal protection *in vivo*. As a central player in the endogenous antioxidant response, GSTP may prove to be an important therapeutic target. Knowledge of the signaling pathways and mechanisms by which GSTP exerts its neuronal protection will most certainly lead to new combined therapeutic strategies that can effectively target multiple aspects of the intricate molecular pathology of Parkinson's disease and other neurodegenerative disorders.

2

GLUTATHIONE S-TRANSFERASE pi MEDIATES MPTP-INDUCED c-JUN N-TERMINAL KINASE ACTIVATION IN THE NIGROSTRIATAL PATHWAY

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2.1 Abstract

Parkinson's disease (PD) is a progressive movement disorder resulting from the death of dopaminergic neurons in the *substantia nigra*. Neurotoxin-based models of PD using 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) recapitulate the neurological features of the disease, triggering a cascade of deleterious events through the activation of the c-Jun N-terminal kinase (JNK). The molecular mechanisms underlying the regulation of JNK activity under cellular stress conditions involve the activation of several upstream kinases along with the fine-tuning of different endogenous JNK repressors. Glutathione S-Transferase pi (GSTP), a phase II detoxifying enzyme, has been shown to inhibit JNK-activated signaling by protein-protein interactions, preventing c-Jun phosphorylation and the subsequent trigger of the cell death cascade.

Here we use C57BL/6 wild-type and GSTP knockout mice treated with MPTP to evaluate the regulation of JNK signaling by GSTP in both the *substantia nigra* and the striatum.

The results presented herein show that GSTP knockout mice are more susceptible to the neurotoxic effects of MPTP than their wild-type counterparts. Indeed, the administration of MPTP induces a progressive demise of nigral dopaminergic neurons together with the degeneration of striatal fibres at an earlier time-point in the GSTP knockout mice when compared to the wild-type mice. Also, MPTP treatment leads to increased p-JNK levels and JNK catalytic activity in both wild-type and GSTP knockout mice midbrain and striatum. Moreover, our results demonstrate that *in vivo* GSTP acts as an endogenous regulator of the MPTP-induced cellular stress response by controlling JNK activity through protein-protein interactions.

2.2 Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by a progressive degeneration of dopaminergic (DA) neurons in the substantia nigra pars compacta (SNpc) and the presence of intracytoplasmic inclusions of aggregated proteins (Lewy bodies, LB) (Dauer and Przedborski 2003). The cellular mechanisms underlying DA cell death in PD are still not fully understood, but several lines of evidence indicate that environmental toxins, genetic factors, mitochondrial dysfunction, oxidative stress and neuro-inflammation are implicated in both familial and sporadic forms of PD (Dauer and Przedborski 2003; Zhou et al. 2008a; Lee et al. 2009; Miller et al. 2009). Mitochondrial inhibitors, such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induce a cascade of deleterious events that recapitulate the neuropathological features of idiopathic PD and culminate with nigral DA neuron degeneration (Zhang et al. 2000; Dauer and Przedborski 2003; Castro-Caldas et al. 2009b). After its systemic administration, MPTP rapidly crosses the blood-brain barrier (Saporito et al. 2000). Once in the brain, MPTP is metabolized in glial cells to 1-methyl-4-phenylpyridinium (MPP^+), the active toxic compound. MPP^+ has high affinity for plasma membrane dopamine transporter, and once inside DA neurons, MPP^+ accumulates in the mitochondria where it potently inhibits complex I of the electron transport system, leading to ATP depletion, loss of mitochondrial membrane potential and the formation of reactive oxygen species (ROS) (Chan et al. 1991; Cassarino et al. 1997; Nicotra and Parvez 2002).

Several studies have demonstrated that c-Jun N-terminal kinase (JNK), a stress-activated protein kinase (SAPK), is a key mediator of MPTP/MPP^+-induced neuronal apoptosis in animal and cellular models of PD (Nishi 1997; Saporito et al. 2000; Gearan et al. 2001; Kuan and Burke 2005), and evidence also points to a role of JNK in the neurodegenerative process that occurs in sporadic PD (Peng and Andersen 2003; Hunot et al. 2004).

The activation of JNK is mediated through a sequential kinase cascade that includes mitogen-activated protein kinase (MAPK) kinase (MAPKK) and MAPK kinase kinase (MAPKKK). JNK is activated by dual phosphorylation of the Thr-Pro-Tyr motif located in the activation loop by MKK4 or MKK7 (Davis 2000; Kuan and Burke 2005). The downstream events of JNK activation leading to apoptosis-mediated cell death

involve both transcriptional and non-transcriptional or mitochondrial mechanisms. In the transcriptional mechanism c-Jun is the critical mediator of the pro-apoptotic effects of JNK. Once phosphorylated by JNK on two sites within the activation domain (Ser63 and Ser73) (Weston and Davis 2002), c-Jun has increased transcriptional activity leading to upregulation of a number of genes involved in the control of cell survival and apoptosis (Peng and Andersen 2003; Kuan and Burke 2005; Silva et al. 2005). The mitochondrial mechanism of JNK signaling involves direct phosphorylation and modulation of pro- and anti-apoptotic activity of Bcl-2 family proteins (Davis 2000; Nicotra and Parvez 2002; Schroeter et al. 2003; Kuan and Burke 2005; Silva et al. 2005).

The assumption that the JNK signaling pathway is an important checkpoint in MPTP/MPP^+-induced neurotoxicity comes from the observation that increased levels of phosphorylated MKK4, JNK and c-Jun precede and are implicated in the degeneration of DA neurons from MPTP-treated mice SNpc (Saporito et al. 2000; Nicotra and Parvez 2002; Peng and Andersen 2003; Kuan and Burke 2005; Silva et al. 2005). Selective JNK inhibitors, such as CEP-1347/KT-7515, SP600125 and small inhibitor peptides also protect against MPTP-induced apoptosis in the nigrostriatal DA neurons *in vivo* (Saporito et al. 1999; Wang et al. 2004; Pan et al. 2010). Moreover, gene targeting studies showed that JNK-null mice have increased resistance to MPTP-induced neuronal cell death in the SNpc (Hunot et al. 2004).

Glutathione S-transferases (GST) are phase II drug metabolizing enzymes that catalyze the conjugation of reduced glutathione to electrophilic groups on substrate molecules, namely by-products of oxidative stress rendering them more soluble and thus more easily eliminated from the cell. There are seven cytosolic subunit classes of GSTs (alpha, mu, pi, theta, omega, sigma and zeta), three microsomal and one mitochondrial form (Salinas and Wong 1999; Board et al. 2000; Hayes et al. 2005). GSTs have generally a ubiquitous localization, while some isoforms show a tissue-specific distribution. In mammals, only alpha, mu and pi isoforms are expressed in the central nervous system (CNS) (Johnson et al. 1993; Shang et al. 2008); the most highly expressed is GSTP, which in addition to detoxification reactions also acts as a ligand-binding protein controlling the catalytic activity of JNK (Adler et al. 1999; Yin et al. 2000; Elsby et al. 2003). The interaction between GSTP and JNK involves the C-terminal region of domain 2 of GSTP and the C-terminal domain of JNK, and requires the monomeric (Adler et al. 1999; Wang et al. 2001) or, as recently suggested, dimeric forms of GSTP

(Gildenhuys et al. 2010). Under conditions of oxidative stress, GSTP dissociates from JNK which may then be phosphorylated and phosphorylate its downstream substrates. Therefore, GSTP may serve as an endogenous regulator of the cellular stress response, eliciting protection against cell death induced by ROS by controlling JNK activity (Adler et al. 1999; Yin et al. 2000).

We and others have reported that in the mouse brain, GSTP is constitutively and predominantly expressed in glial cells (namely oligodendrocytes and astrocytes) and also in DA neurons from SNpc (Johnson et al. 1993; Beiswanger et al. 1995; Martinez-Lara et al. 2003; Smeyne et al. 2007; Castro-Caldas et al. 2009b; Shi et al. 2009). In a previous study a single dose of MPTP in C57BL/6 mice induced the transient overexpression of GSTP particularly in glial cells from both midbrain and striatum (Castro-Caldas et al. 2009b). Moreover, we have also demonstrated that in SH-SY5Y cells GSTP interacts with JNK, suggesting that in neuronal cells GSTP directly modulates JNK catalytic activity and cell death (Castro-Caldas et al. 2009a).

An association between *GSTP1* genotypes and sporadic forms of PD was described in different groups of patients exposed to pesticides (Menegon et al. 1998), and GST polymorphisms have been shown to increase DA neuron loss in a parkin mutant *Drosophila* model (Whitworth et al. 2005). Importantly, data from our group has shown that subjects who carry the GSTP1*B allele may be at a significantly increased PD risk, strongly suggesting a neuroprotective role for native *GSTP1* (Vilar et al. 2007).

In this work we used MPTP-treated C57BL/6 wild-type and GSTP knockout (GSTP ko) mice to further evaluate the role of GSTP in this *in vivo* model of PD. We show that GSTP ko mice are more susceptible to MPTP neurotoxicity than wild-type mice, and suggest that GSTP exerts its neuroprotective role through direct regulation of JNK catalytic activity.

2.3. Materials and Methods

2.3.1 Materials

MPTP, mouse anti-tyrosine hydroxylase (TH) (T2928) antibody, Hoechst 33258, amido black and the GST assay kit were purchased from Sigma Chemical Co (St Louis, MO, USA). Mouse anti-GSTP antibody (G59720) was from BD Biosciences Pharmingen (San Jose, CA, USA). The DakoCytomation Fluorescent Mounting Medium was from Dako (Copenhagen, Denmark). The non-radioactive SAPK/JNK assay kit (#9810), the rabbit anti-JNK (#9258) and mouse anti-p-JNK (Thr183/Tyr185) (#9255) antibodies, the horseradish peroxidase (HRP)-conjugated anti-mouse IgG (#7076) and the anti-c-Jun antibodies (#9260) were from Cell Signaling Technology (Beverly, MA, USA). The Tyramide Signal Amplification Kit was purchased from Invitrogen/Molecular Probes (Eugene, OR, USA), the Protein A/G PLUS-Agarose beads and the anti-α-tubulin antibody (B-7) (sc-5286) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-rabbit secondary antibody (W401B) was from Promega (Madison, USA). Immobilon P was from Millipore (Bedford, MA, USA). ECL and Hyperfilm ECL were purchased from Amersham Biosciences (Piscataway, NJ, USA). Other chemical and reagents were of the highest analytical grade and were purchased from local commercial sources.

2.3.2 Animals and treatment

All procedures were carried out in accordance with National Institutes of Health guidelines for the care and use of animals and methods were approved by the local Institutional Animal Care and Use Committee.

Twelve week-old male wild-type C57BL/6 mice were purchased from the Gulbenkian Institute of Science Animal House (Oeiras, Portugal). C57BL/6 *Gstp1/p2* null mice from Cancer Research UK were kindly provided by C. Roland Wolf and this null lineage was re-derived and also maintained at the same Animal House. The work we report here his based on this double-knockout line, since in the mouse both Gstp genes (*Gstp1* and *Gstp2*) are arranged in tandem on chromosome 1 and were deleted by homologous recombination (Henderson et al. 1998b). Throughout the text and figures this knockout mice line will be referred as GSTP knockout (GSTP ko). All animals were

housed under standardized conditions on a 12 hours light-dark cycle with free access to a standard diet and water *ad libitum*.

MPTP was administered intra-peritoneally at a single dose of 40 mg/kg (Saporito et al. 2000; Castro-Caldas et al. 2009b). Control mice received saline alone. At the indicated times after neurotoxin or vehicle administration, mice were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), decapitated and brains were quickly removed and placed in fresh PBS. Mice brains were then placed on their ventral surface onto a mouse brain matrice (Agar Scientific), and a slice between Bregma -2.5 and Bregma -3.8 was isolated. This removed brain slab was placed flat and the entire midbrain region, containing the SNpc was dissected as previously described (Smeyne and Smeyne 2002). The remaining brain piece was then cut between Bregma –1.0 and 1.5, and whole striatum was isolated after discarding the cerebral cortex, the corpus callosum and the region of the septum (including septohippocampal bundle, lateral septal nucleus, medial septal nucleus and diagonal band). The specific pieces of interest were flash frozen under liquid nitrogen and kept at -80ºC until further use.

The time course studies were carried out in three independent experiments (n=3) with groups of three to six mice.

2.3.3 Immunohistochemistry

After MPTP treatment mice were anesthetized, transcardially perfused and processed for cryostat sectioning. Brain sections at the level of SNpc (Bregma -3.20) and at the level of midstriatum (Bregma 1.00) were obtained, as previously described (Castro-Caldas et al. 2009b). TH immunolocalization was performed with the Tyramide Signal Amplification Kit according to the manufacturer's instructions. Briefly, 14 μm thick coronal sections were permeabilized with 0.2% Triton X-100 in PBS, and then pre-treated with blocking solution (2% bovine serum albumin, 0.05% Tween-20 in PBS). Incubation with the anti-TH antibody (1:250) was performed overnight at 4ºC, followed by incubation with the HRP-conjugated goat anti-mouse IgG for 30 min, and subsequently with the Alexa Fluor 488-labeled tyramide for 10 min at room temperature. Sections were mounted in fluorescent mounting medium containing 5 μg/mL Hoechst 33258, observed under an Axioskop microscope (Carl Zeiss) with an attached Leica DFC490 camera, and photographed using Image Manager 50 software (Leica Microsystems, Inc.). The

specificity of the primary antibody used was previously confirmed by Western blot analysis. Control experiments for non-specific binding were performed in parallel by omission of the primary and secondary antibodies.

The number of nigral TH-positive cells was counted in 4 adjacent sections, using 3 animals per experiment, and averaged.

2.3.4 Western Blot Analysis

Tissue extracts from mice midbrains and striata were prepared as indicated elsewhere (Castro-Caldas et al. 2009b), and were resolved on 12% SDS-PAGE, and electro-transferred onto Immobilon P. The membrane was blocked with 5% non-fat dry milk in Tris-buffered saline with 0.1% Tween-20 and further incubated with the anti-phospho-JNK antibody (1:500), anti-phospho-c-Jun (1:1000), overnight at 4ºC, followed by incubation with HRP-conjugated anti-mouse and anti-rabbit antibodies. The immunocomplexes were detected by the ECL chemiluminescent method and visualized with Hyperfilm ECL. Analysis of total JNK (1:1000) and α-tubulin (1:15000) expression was performed in stripped membranes as a loading control. The relative intensities of protein bands were analyzed using the Gel-Pro 32 Analyzer densitometry analysis software (Media Cybernetics, MD, USA).

2.3.5 Measurement of JNK catalytic activity

JNK catalytic activity was determined as previously described (Elsby et al. 2003) by measuring the levels of c-Jun phosphorylation using the non-radioactive SAPK/JNK assay kit, according to the manufacturer's instructions. Briefly, the kinase reaction was performed in the presence of excess ATP and was optimized for both time (30 min) and protein concentration (1 mg of total protein tissue extract). Samples were electrophoresed on 12 % SDS-PAGE and electroblotted onto Immobilon P. c-Jun phosphorylation was selectively measured using an anti-phospho-c-Jun antibody (1:1000), which specifically measures JNK-catalysed phosphorylation of c-Jun at Ser-63. To demonstrate equivalent protein loading, the membranes were stripped and then incubated with anti-c-Jun antibody (1:5000).

2.3.6 Co-immunoprecipitation Assays

Co-immunoprecipitations were carried out using 1mg of total protein extracts from striatum or midbrain incubated with 4 μl of the anti-JNK antibody, overnight at 4ºC. Then, 20 μl of protein A/G PLUS-Agarose beads were added for 2 hours, at 4ºC. Co-immunoprecipitation complexes were ressuspended in washing buffer (50mM Tris-HCl pH 7.4, 180mM NaCl, 1mM EDTA, 1% Triton-X 100) and recovered by centrifugation at 12000 *g* for 10 minutes at 4 ºC. The washing process was repeated three times. Samples were boiled in denaturating buffer (0.25 mM Tris-HCl, pH 6.8, 4% SDS, 40% glicerol, 0.2% bromophenol blue, 1% β-mercaptoethanol). The presence of the protein immuno-complexes was evaluated by 12% SDS-PAGE followed by immunoblotting using the anti-GSTP antibody (1:1000). As a control, JNK was detected in the same membrane after stripping off the immune complex for the detection of GSTP. Immunoblot analysis showed an absence of nonspecific binding of the JNK antibody to GSTP. In addition, control experiments for non-specific binding were performed by immunoblot analysis of immunoprecipitation assays using an antibody reactive to Bcl-2.

2.3.7 Statistical analysis

All results are expressed as mean ± SEM values. Data were analyzed by the one-way ANOVA, and differences between groups were determined by Tukey post test (Graphpad, Prism 5.0, San Diego, CA, USA). Means were considered statistically significant at a *p* value below 0.05.

2.4 Results

2.4.1 MPTP-induced DA cell loss in the nigrostriatal pathway

To determine whether endogenous GSTP has a role in MPTP-dependent nigrostriatal DA cell loss, we examined the effects of the neurotoxin on SNpc and striatal TH immunopositive cells and fibres, respectively, in wild-type and in GSTP ko mice.

We have previously shown that MPTP induces an evident depletion of DA fibres and cells of the nigrostriatal pathway from wild-type C57BL/6 animals by a "dying back" mechanism.

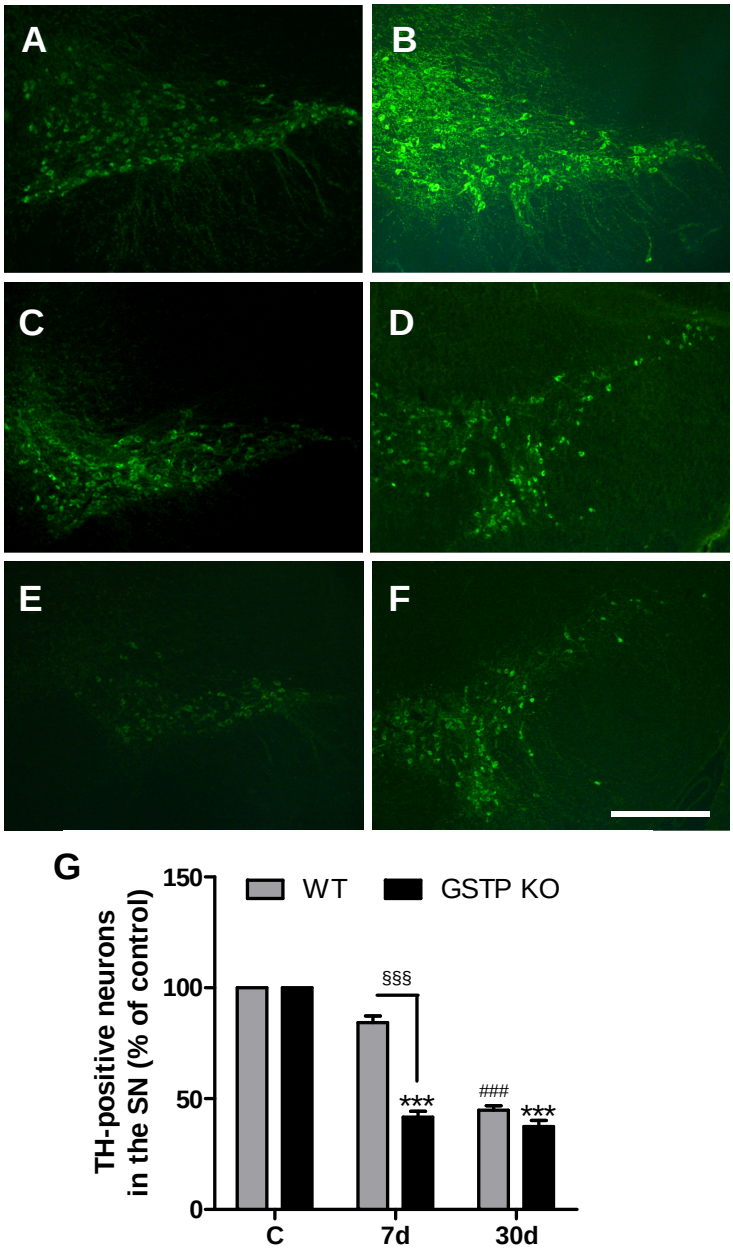


Figure 2.1. Nigral dopaminergic neurons degeneration after MPTP administration. Dopaminergic neurons were identified by TH immunostaining in coronal sections at the level of SNpc (Bregma –3.20) from C57BL/6 wild-type (WT) (**A**, **C** and **E**) and GSTP knockout (KO) (**B**, **D** and **F**) mice. (A and B) Saline-treated (control); (C and D) 7 days and (E and F) 30 days post-MPTP administration; (**G**) The values shown are the averaged number of TH positive neurons in the *zona compacta* of the SN counted in 4 adjacent sections ± SEM of three independent experiments, ###p<0.001 *vs* WT control; ***p<0.001 *vs* KO control; $^{§§§}p$<0.001 *vs* WT (7d). Microphotographs shown are representative of three independent experiments. Scale bar = 100 µm

Results presented in Figure 2.1 show that the loss of DA cells in the SNpc from wild-type mice was evident and significant 30 days after MPTP administration, whereas MPTP induces a significant decrease of about 60% in the number of nigral DA neurons from C57BL/6 GSTP ko mice, 7 days after i.p. injection (one-way ANOVA with Tukey post-hoc test: F=251.1 df=2, p<0.001). An equivalent DA cell loss was detected in GSTP ko mice sacrificed 30 days after MPTP administration (one-way ANOVA with Tukey post-hoc test: F=251.1 df=2, p<0.001).

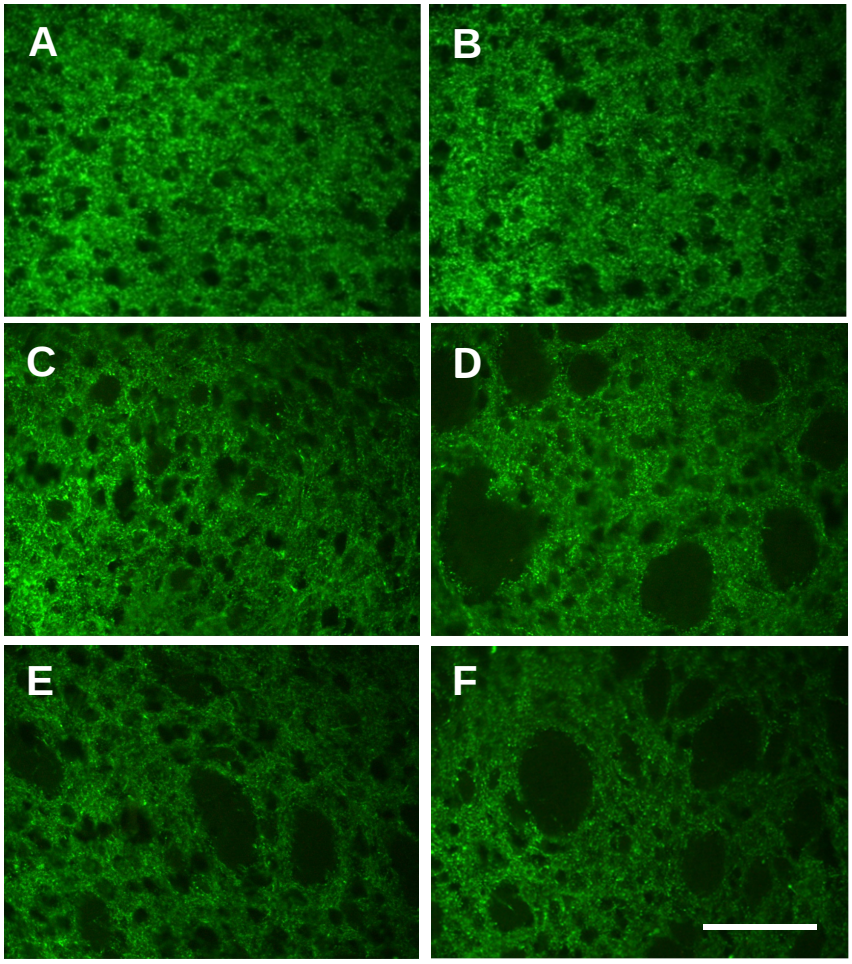


Figure 2.2. Striatal dopaminergic fibres degeneration after MPTP administration. Dopaminergic fibres were identified by TH immunostaining in coronal sections at the level of midstriatum (Bregma 1.00) from C57BL/6 wild-type (**A**, **C** and **E**) and GSTP knockout (**B**, **D** and **F**) mice. (A and B) Saline-treated (control); (C and D) 7 days; (E and F) 30 days post-MPTP administration, respectively. Microphotographs shown are representative of three independent experiments. Scale bar = 100 µm

Representative sections from the midstriatum, presented in Figure 2.2, show that MPTP administration resulted in a dramatic reduction of TH positive fibres density as early as 7 days after the neurotoxic insult in GSTP ko mice, relatively to its own control (compare panel D with panel B) and also to the corresponding results from wild-type animals (compare panel D with panel C).

DA fibre degeneration in striata from GSTP ko, as well as wild-type mice was significantly evident 30 days after MPTP administration. By that time point, TH-positive striatal fibres density and immunofluorescence intensity showed a similar pattern in striata from both wild-type and GSTP ko mice (Figure 2.2: panels E and F).

No significant difference in the number and density of TH positive cells and fibres was observed in the brain slices from mice sacrificed before day 7 post-MPTP administration (data not shown). Moreover, these results also indicate that 30 days after MPTP administration the level of DA cell death is equivalent in both wild-type and GSTP ko mice.

Taken together, these results show that in GSTP ko mice the MPTP-induced DA cell loss occurs earlier, indicating that these mice are more susceptible to MPTP toxicity.

2.4.2 MPTP-induced JNK phosphorylation and activation

To determine whether endogenous GSTP affects the profile of JNK activation in the presence of MPTP, the phosphorylation status as well as the catalytic activity of JNK was evaluated in striata and midbrains from wild-type and GSTP ko mice treated with MPTP.

JNK phosphorylation was evaluated by Western blot assay using a specific antibody that recognizes dual phosphorylation of JNK (Thr183/Tyr85). Figure 2.3 shows that 3h post MPTP administration, the levels of phosphorylated JNK are significantly increased in striata (Figure 2.3A) and midbrains (Figure 2.3B) from wild-type and GSTP ko animals.

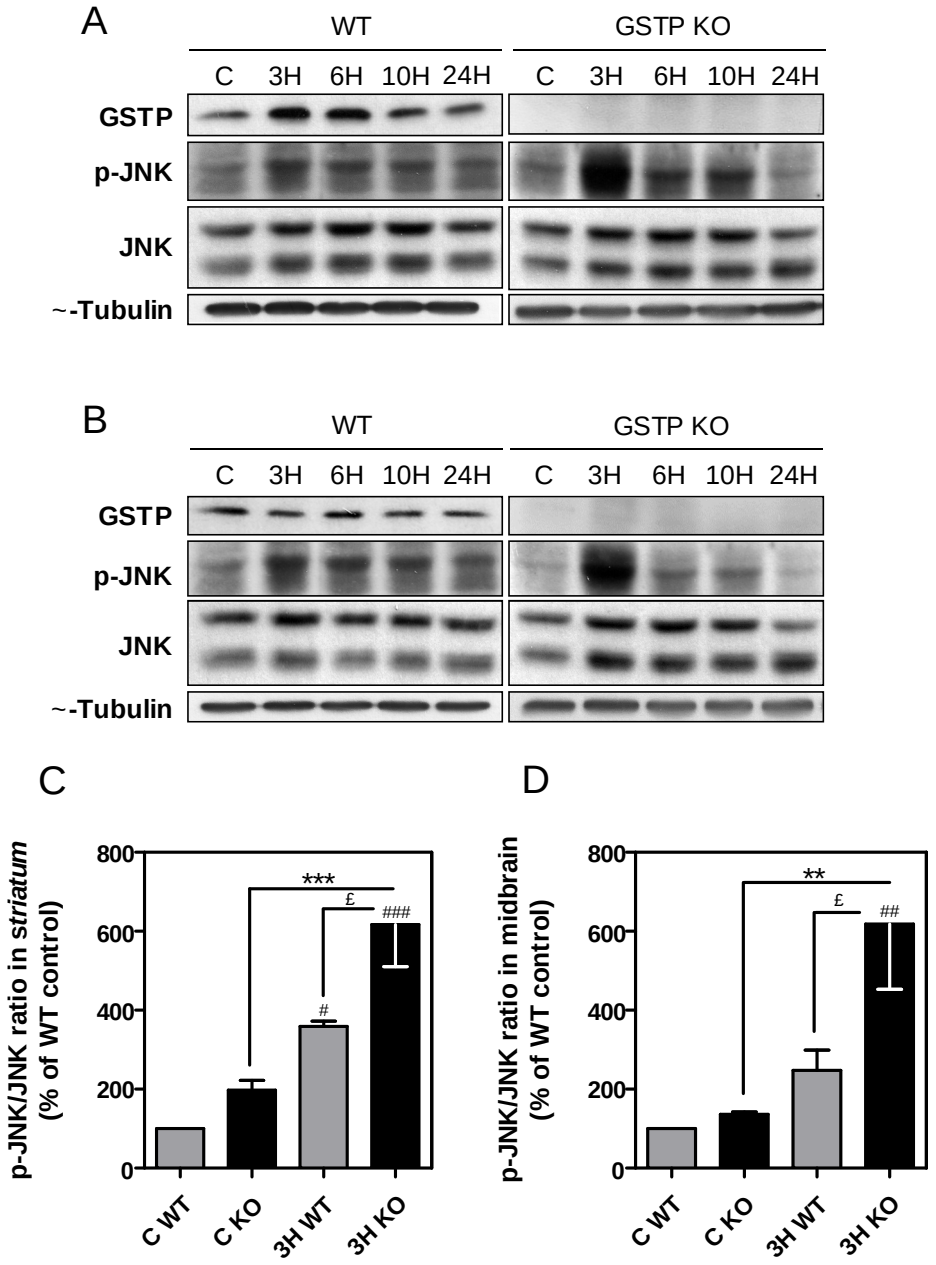


Figure 2.3. JNK phosphorylation levels in response to MPTP administration in wild-type and GSTP knockout mice. Tissue extracts from C57BL/6 wild-type (WT) and GSTP knockout (GSTP KO) mice striatum (**A**) and midbrain (**B**) were prepared after saline (control, C) or MPTP single dose (40 mg/ Kg body weight) i.p. injection. Mice were sacrificed 3 h, 6 h, 10 h and 24 h after MPTP administration. Tissue extracts were subjected to SDS/PAGE, and the corresponding blots were probed with antibodies to GSTP, p-JNK, JNK and α-tubulin. The phospho-JNK/ JNK ratios from striatum (**C**) and from midbrain (**D**) at 3h time-point from WT and GSTP KO mice were plotted as the mean ± SEM of three independent experiments, indicated as percentage of controls from wild-type animal samples, #p<0.05, ##p<0.01 and ###p<0.001 *vs* WT control; $^{**}p$<0.01 and $^{***}p$<0.001 *vs* KO control; $^{£}p$<0.05 *vs* WT (3h).

The observed increases of the JNK phosphorylation status were shown to be both fast and transient since p-JNK control levels are restored 6h after MPTP administration, in both brain regions and mouse lines.

Interestingly, the phosphorylation level of JNK 3h after MPTP treatment was more evident and statistically different in striata from GSTP ko mice as compared with both controls and the corresponding sample from wild-type mice (Figure 2.3C) ($p<0.05$ and $p<0.001$, one way ANOVA with Tukey post-hoc test).

To further elucidate the effect of endogenous GSTP on the JNK pathway, a non-radioactive kinase assay for measuring JNK activity was performed. In this assay phosphorylation of the downstream substrate c-Jun was utilized to determine JNK enzymatic activity in the studied brain regions from wild-type and GSTP ko mice.

There was a transient increase in the amount of *in vitro* phosphorylated c-Jun, reaching a peak 3h or 6h following MPTP administration, in striata (Figure 2.4A and 2.4C), and 3h or 6h in midbrains (Figure 2.4B and 2.4D) from GSTP ko and wild-type mice, respectively. Although the results from wild-type and GSTP ko mice, shown in Figure 4, are presented as a percentage of their respective controls, it is worth noticing that the ability of JNK to *in vitro* phosphorylate c-Jun is significantly higher in the striatum and midbrain control samples from GSTP ko mice.

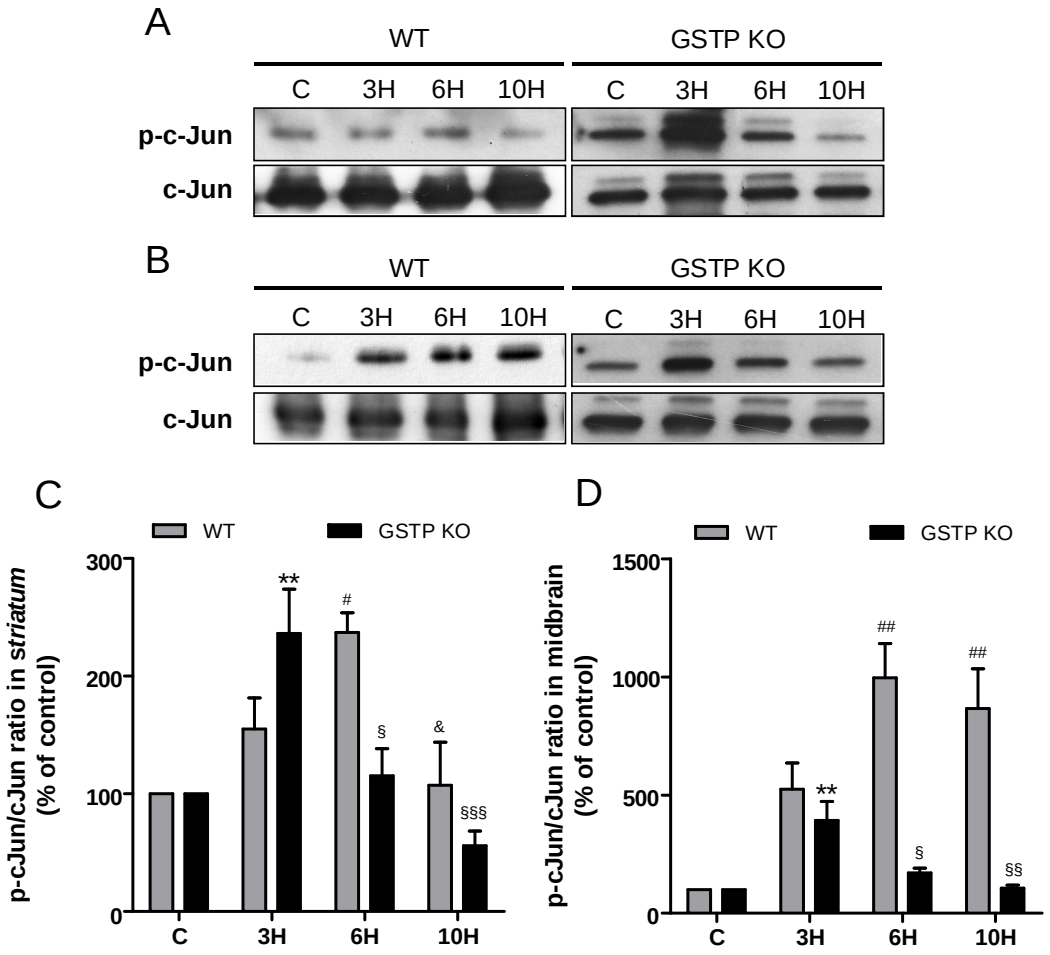


Figure 2.4. JNK activation after MPTP administration in wild-type and GSTP knockout mice. Tissue extracts from C57BL/6 wild-type (WT) and GSTP knockout (GSTP KO) mice striatum (**A**) and midbrain (**B**) were prepared after saline (control, C) or MPTP single dose (40 mg/ Kg body weight) i.p. injection. Mice were sacrificed 3 h, 6 h, and 10 h after MPTP administration. JNK enzymatic activity was evaluated with a non-radioactive kinase assay: tissue extracts were incubated with c-Jun fusion beads to pull-down JNK. Kinase reactions were then performed, *in vitro* and the phosphorylation of c-Jun was assessed by immunoblotting. The blots were probed with antibodies to phospho-c-Jun (p-c-Jun) and c-Jun. The immunoblots presented are representative of three independent experiments. The p-c-Jun/c-Jun ratios from striatum (**C**) and from midbrain (**D**) are given as mean ± SEM of three independent experiments, $^{**}p<0.01$ *vs* KO control; $^{\#}p<0.05$ and $^{\#\#}p<0.01$ *vs* WT control; $^{\S}p<0.05$, $^{\S\S}p<0.01$ and $^{\S\S\S}p<0.001$ *vs* KO (3h); $^{\&}p<0.05$ *vs* WT (6h).

In vivo phosphorylation of c-Jun was also assessed by Western blot assay using a specific antibody that recognizes phosphorylation of c-Jun (Ser73). Results presented in Figure 2.5 show that in the evaluated time points no significant increase in the levels of p-c-Jun were detected in both midbrain and striata from wild-type mice. However, the

endogenous c-Jun is significantly activated in the midbrain of GSTP ko mice at 6 hours following MPTP administration.

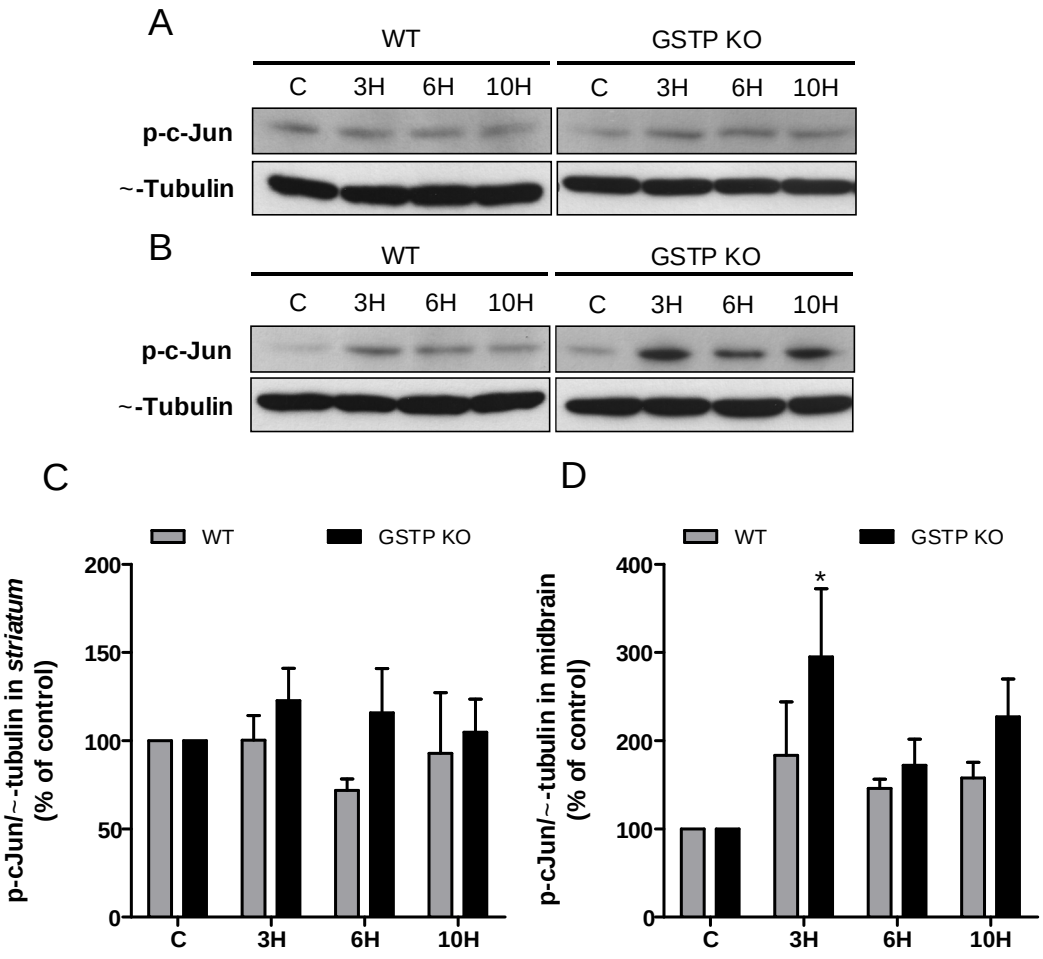


Figure 2.5. p-c-Jun protein expression in wild-type and GSTP knockout mice following MPTP treatment. Tissue extracts from C57BL/6 wild-type (WT) and GSTP knockout (GSTP KO) mice striatum (**A**) and midbrain (**B**) were prepared after saline (control, C) or MPTP single dose (40 mg/ Kg body weight) i.p. injection. Mice were sacrificed 3 h, 6 h and 10 h after MPTP administration. Tissue extracts were subjected to SDS/PAGE, and the corresponding blots were probed with antibodies to phospho-c-Jun (p-c-Jun) and α-tubulin. The immunoblots presented are representative of three independent experiments. The p-c-Jun/α-tubulin ratios from striatum (**C**) and from midbrain (**D**) are given as mean ± SEM of three independent experiments, **p*<0.05 *vs* KO control.

2.4.3 GSTP modulation of JNK activity by physical interaction *in vivo*

GSTP was characterized as a JNK-associated protein exerting its inhibitory effects on the kinase activation by physical protein-protein interaction (Adler et al. 1999).

To elucidate the role of GSTP on the regulation of JNK activity upon MPTP administration we performed co-immunoprecipitation assays to evaluate putative alterations of GSTP-JNK protein-protein complex in striatal and midbrain samples from wild-type mice.

The results of JNK-GSTP pull-down assays, presented in Figure 2.6, show that these two proteins physically interact *in vivo*, and that this complex is present in both tissue extracts from control mice.

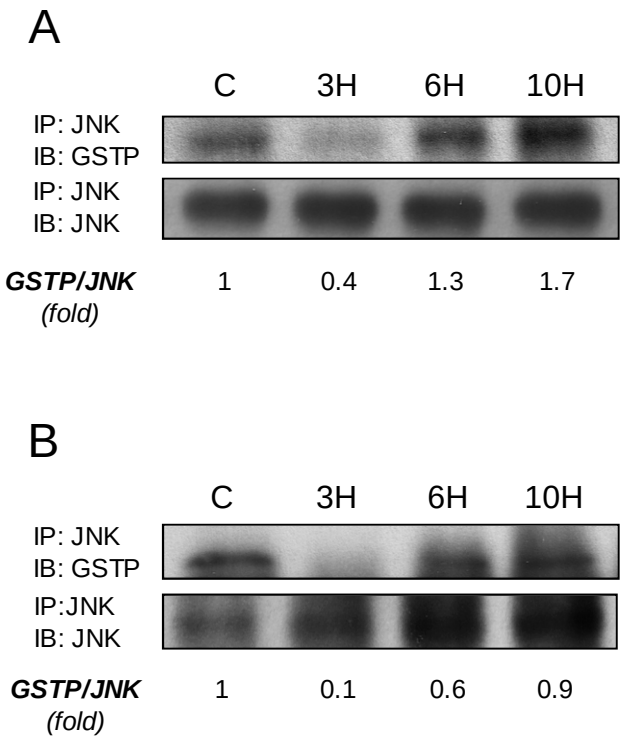


Figure 2.6. Determination of protein-protein interaction between GSTP and JNK after MPTP treatment. Tissue extracts from C57BL/6 wild-type mice striatum (**A**) and midbrain (**B**), before (control, C) or 3 h, 6 h and 10 h after MPTP administration were prepared. Protein-protein interactions between GSTP and JNK were evaluated by co-immunoprecipitation assays (IP) using the anti-JNK antibody followed by immunoblotting (IB) with the antibody to GSTP. The immunoblots presented are representative of three independent experiments.

Interestingly, treatment with MPTP induces the dissociation of this complex 3 h post-treatment in both striatum and midbrain. The protein complex dissociation seems to be transient since 10h post-MPTP administration GSTP-JNK association levels return to a value comparable to the control. Remarkably, the time points in which the transient increase of JNK enzymatic activity occurs correlate with the dissociation of GSTP-JNK complex, indicating that in the absence of GSTP, JNK is free to be phosphorylated and to phosphorylate its downstream substrates. Moreover, the re-association between GSTP and JNK occurs at time points when we have previously described an increased expression of GSTP following MPTP administration (Castro-Caldas et al. 2009b), suggesting that the increase in the pool of GSTP restores JNK to an inactive state by protein-protein interaction.

2.5 Discussion

GSTP belongs to the phase II family of detoxification enzymes and plays a critical role in protection against oxidative stress by either conjugation of glutathione (GSH) to electrophilic substrates or by direct regulation of the stress kinase JNK pathway (Mannervik and Danielson 1988; Hayes et al. 2005; Henderson and Wolf 2011). Our present data are novel in that they provide experimental evidence that, *in vivo*, one mechanism by which GSTP exerts its neuroprotective actions is by physical association with JNK leading to down-regulation of kinase activity.

Results presented herein show that administration of MPTP induces a progressive demise of nigral DA neurons in parallel with the degeneration of striatal terminals in both wild-type and GSTP ko mice. However, while in GSTP ko mice DA cell death in SNpc is significant 7 days post-MPTP, in wild-type animals it only reaches significance 30 days post-MPTP administration as we had previously demonstrated (Castro-Caldas et al. 2009b). In accordance, the degeneration of striatal fibres also occurs at an earlier time point in GSTP ko mice as compared with wild-type mice. These results indicate that GSTP ko mice are more susceptible to MPTP neurotoxic effects than wild-type mice. Since 30 days after MPTP both groups of animals show equivalent DA neurodegeneration, we hypothesise that endogenous GSTP exerts its neuroprotective effects against MPTP toxicity through the modulation of upstream events in the degenerative cascade.

MPTP administration causes the inhibition of mitochondrial respiration which results in rapid secondary events, including depletion in intracellular ATP and increased levels of oxidative stress (Chan et al. 1991; Cassarino et al. 1997). We previously confirmed the presence of increased markers of oxidative stress following MPTP treatment in both wild-type and GSTP ko mice (data not shown). Oxidative stress is also an important activator of the JNK signaling cascade. In fact, we have shown that MPTP administration induces a fast and transient increase in the levels of phosphorylated JNK in striata and midbrains from wild-type and GSTP ko mice. Moreover, we demonstrate that upon MPTP treatment JNK is transiently activated and capable of phosphorylating its downstream substrate c-Jun.

Constitutively increased JNK activity has been previously described in the liver, lung and fibroblasts from GSTP ko mice (Adler et al. 1999; Elsby et al. 2003). In agreement, our results show that in striata the basal levels of phosphorylated JNK are higher in GSTP ko mice. Importantly, following MPTP treatment the levels of phosphorylated JNK are significantly higher in GSTP ko mice as compared with the wild-type counterparts.

Given the extensive experimental evidence showing that JNK signaling pathway mediates part of the cellular mechanisms operating in neurodegenerescence induced by MPTP (Saporito et al. 1999; Hunot et al. 2004; Wang et al. 2004; Pan et al. 2010), we believe that the significant difference in the in vivo activity of JNK, between GSTP ko and wild-type mice, might explain their different susceptibility to MPTP.

It is now becoming evident that GSTP may have important cellular actions that do not solely involve its primary catalytic functions. GSTP regulates intracellular signaling pathways as an endogenous inhibitor of JNK and can have a non-enzymatic regulatory role in controlling cellular response to external stimuli (Davis et al. 2001).

Interestingly, by co-immunoprecipitation assays we demonstrate that GSTP physically interacts with JNK in mice brain extracts from striatum and midbrain. Moreover, these assays also indicate that the kinetics of GSTP-JNK dissociation in response to MPTP reflects the time course of JNK activation and the upregulation of GSTP expression following MPTP intoxication. In untreated control mice, GSTP is bound to JNK, keeping the kinase in an inactive state. MPTP administration induces a significant transient increase in JNK phosphorylation and activation, which is accompanied by the release of GSTP. In parallel with the increased activation of JNK we

have previously shown that GSTP expression is induced by MPTP (Castro-Caldas et al. 2009b). This explains, at least in part, the transient activation of JNK, since 10 hours after MPTP administration GSTP-JNK complexes return to levels comparable to the control. GSTP has been shown to directly bind to JNK in several cell types and to negatively regulate JNK pathway under different stress conditions (Adler et al. 1999; Wang et al. 2001; Gildenhuys et al. 2010). For example, using human SH-SY5Y neuroblastoma cell cultures, we have previously shown that, *in vitro,* GSTP forms a complex with JNK, therefore maintaining the catalytic kinase activity low in the absence of a stress stimulus. UV light triggered the dissociation of the complex liberating JNK to be phosphorylated and to phosphorylate c-Jun (Castro-Caldas et al. 2009a). However, as far as we know, this is the first time that the interaction between GSTP and JNK is demonstrated to occur *in vivo*.

Previously, we have identified DA neurons, but mainly astrocytes and oligodendrocytes as GSTP positive cells, in midbrain and striatum from C57BL/6 wild-type mice (Castro-Caldas et al. 2009b). Interestingly, upregulation of the JNK pathway has been demonstrated not only in DA neurons following MPTP administration, but also in glial cells in a neurodegenerative environment.

There is now increasing recognition for the role of astrogliosis and activated microglia as major factors in the pathogenesis of PD, and also in the neurodegenerative process that occurs in the presence of MPTP/MPP^+ (Kohutnicka et al. 1998; McGeer and McGeer 2008; Lee et al. 2009). Neuronal inflammation is characterized by glial morphological changes, increased expression of surface molecules, secretion of soluble pro-inflammatory mediators, upregulation of cyclooxygenase-2 and nitric oxide synthase and phagocytosis of degenerating neurons (Teismann et al. 2003; McGeer and McGeer 2008; Lee et al. 2009). Thus, once becoming activated by injured neurons, glia can turn into a source of damaging toxic factors to inflict harm to other neurons and glial cells in the vicinity. For example, oligodendrocytes are extremely vulnerable to increases in ROS and pro-inflammatory mediators produced by activated microglia and astrocytes. Oligodendrocyte cell death results in extensive demyelination which further contributes to neuron degeneration (Taylor et al. 2010). On the other hand, astrogliosis and microgliosis have a parallel paracrine effect, inducing cell death in astrocytes and microglia, playing a role in a self-amplifying cycle of degeneration. Glial death induced by neuroinflammation

requires the activation of JNK intracellular signaling pathway (Jurewicz et al. 2003; Jurewicz et al. 2006).

To further characterize the neuroprotection elicited by endogenous GSTP against MPTP intoxication, we evaluated total GST catalytic activity (Supplementary Figure 1). We found no differences between total GST specific activity in striata and midbrains from wild-type and GSTP ko mice, neither before nor after MPTP administration. These results indicate that probably there is a compensatory increase in other brain GST isoforms (alpha and mu) in GSTP ko mice; however the protective role of endogenous GSTP in wild-type mice does not seem to depend on its catalytic activity but rather on its ability to inhibit JNK activation.

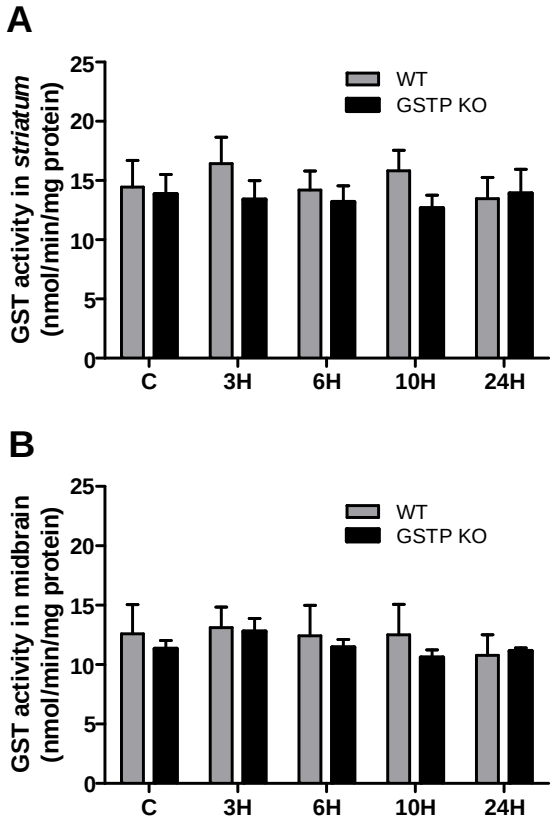
Taken together, the findings presented in this work indicate that the neuroprotective role of GSTP involves the direct regulation of JNK pathway, which is an early event in the degenerative cascade triggered by MPTP. It is likely that the increase in GSTP previously observed in wild-type mice is an adaptive mechanism in response to the neurotoxic and oxidative insults. This hypothesis is corroborated by previous studies showing that an increase in GSTP expression occurs in response to ROS-generating agents and is mediated via the JNK/c-Jun cascade, in a feedback regulatory loop (Xia et al. 1996; Ainbinder et al. 1997; Yin et al. 2000).

It is worth noting that even in GSTP ko mice the phosphorylation and activation of JNK in response to MPTP is also transient, indicating that other factors besides GSTP regulate the catalytic kinase activity. In fact, it has also been shown that the heat shock protein, Hsp70, is another intracellular protein that inhibits JNK signaling *in vitro* and *in vivo* (Park et al. 2001). Interestingly, preliminary results from our work point to a transient increase in heat shock proteins expression in mice brain under MPTP treatment (data not shown). Therefore, Hsp70 appears as a putative candidate that, in the absence of GSTP, may contribute to the negative regulation of JNK activation in the GSTP ko mice.

Finally, GSTP may also exert neuroprotective effects against MPTP-triggered oxidative stress by S-glutathionylation of several proteins. This constitutes a reversible mechanism by which a dissulfide bond is established between a protein cysteinyl residue and glutathione (GSH), protecting proteins from oxidative damages (Townsend 2007). Protein S-glutathionylation following MPTP systemic administration is currently under investigation in our laboratory.

In this present study we provide evidence that GSTP has a neuroprotective role through a non-catalytic, ligand-binding activity with JNK. We consider that drug targeting of the cellular GSTP levels is a potential therapeutic approach for PD intervention.

Supplementary Figure 1



Supplementary Figure 1 GST catalytic activity after MPTP administration. Total GST specific activity was measured by assaying the conjugation of CDNB with GSH in striatum (**A**) and midbrain (**B**) tissue extracts from saline-treated control or MPTP treated C57BL/6 wild-type and GSTP knockout mice. Data shown are the mean values± SEM of three independent experiments, $p>0.05$

Supplementary Material and Methods

GST catalytic activity assay

Total GST enzymatic activity was evaluated in tissue extracts prepared from control and MPTP-treated mice midbrains and striata, using a GST assay kit according to the manufacturer's instructions. GST catalyze the reaction to conjugate glutathione to the 1chloro-2,4-dinitrobenzene (CDNB) substrate through the thiol group of the glutathione. Briefly, the reaction mixture (200µL) contained 5µL of midbrain or striatum homogenate and 2 mM reduced GSH, in PBS. The reaction was initiated by addition of CDNB (final concentration 1mM) at 25ºC. The increase in absorbance at 340 nm, due to the formation of the reaction product (GS-DNB conjugate) was recorded at 60 s intervals for 20 min.

The rate in the UV absorption is directly proportional to the GST activity in the sample. Each assay was performed in duplicate, and enzyme activity was recorded as nmol/min/mg protein.

Acknowledgments

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3

IN VIVO S-GLUTATHIONILATYON OF KEAP1 IN RESPONSE TO MPTP-INDUCED OXIDATIVE STRESS: ROLE OF GLUTATHIONE S-TRANSFERASE pi

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Manuscript submitted

3.1 Abstract

***Aims*:** Glutathione S-transferase pi (GSTP) expression is regulated by the nuclear factor-erythroid 2-related factor 2 (Nrf2) and plays an important defensive role against the accumulation of reactive metabolites that contribute to dopaminergic cell damage.

We hypothesized that in response to oxidative stress, increased GSTP expression may potentiate Keap1 *S*-glutathionylation, leading to Nrf2 activation and neuronal protection.

***Results*:** MPTP-induced oxidative stress leads to glutathione depletion and altered glutathione-dependent reactions including *S*-glutathionylation. Results herein show that GSTP potentiates Keap1 *S*-glutathionylation in mice brain following MPTP administration with subsequent Nrf2 pathway activation, and increased expression of GSTP, in a positive feedback regulatory loop.

***Innovation*:** Our data are novel in that they provide experimental evidence that Keap1 is *S*-glutathionylated *in vivo* in a GSTP-dependent manner.

***Conclusion*:** The presented results unravel a new mechanism contributing to GSTP-elicited neuronal protection.

3.2 Introduction

Parkinson's disease (PD) is a neurodegenerative movement disorder characterized by selective and progressive loss of mesencephalic nigral dopaminergic neurons and the presence of intracytoplasmic inclusions of aggregated proteins (Lewy bodies) (Dauer and Przedborski 2003). Oxidative stress, mitochondrial dysfunction, failure of the proteolytic pathways and neuroinflammation are critical players in the pathogenesis of PD, all of which implicated in reactive oxygen species (ROS) formation (Moore et al. 2005).

The formation of ROS is counter-balanced by appropriate antioxidant defense mechanisms consisting of antioxidant enzymes whose transcription is tightly controlled by binding of the nuclear factor-erythroid 2-related factor2 (Nrf2) to the 5'-upstream regulatory antioxidant response element (ARE) regions of target genes (Lee and Johnson 2004; de Vries et al. 2008).

The Nrf2-ARE signaling pathway plays a significant role in protecting cells from endogenous and exogenous stresses. Nrf2 activity is controlled, in part, by the cytosolic protein Kelch ECH associating protein 1 (Keap1), that under physiological conditions sequesters Nrf2 in the cytoplasm driving it for proteasomal degradation (Kobayashi et al. 2004; Lee and Johnson 2004; Kobayashi et al. 2006; Kensler et al. 2007). Oxidative and electrophilic challenges disrupt the Nrf2-Keap1 complex by modification of reactive cysteine residues of Keap1, thereby allowing Nrf2 to escape proteasomal degradation and to translocate to the nucleus (Kobayashi et al. 2004; Lee and Johnson 2004; Kobayashi et al. 2006; Kensler et al. 2007) where it up-regulates target genes that enhance cell survival.

Glutathione S-transferases (GST; EC 2.5.1.18) are among the genes regulated by Nrf2 (Ikeda et al. 2002; Hayes et al. 2005). GST belong to a multigene family of isoenzymes responsible for the detoxification of electrophiles by conjugation with the nucleophilic thiol-reduced glutathione (GSH) (Ketterer 2001; Hayes et al. 2005). Glutathione S-transferase pi (GSTP) has been shown to protect neurons from ROS by altering the levels of glutathione (Baez et al. 1997; Tew and Ronai 1999), by acting as an endogenous regulator of c-Jun N-terminal kinase (JNK) catalytic activity (Adler et al. 1999; Wang et al. 2001; Castro-Caldas et al. 2012), and by modulating *S*-glutathionylation of proteins (Klatt and Lamas 2000; Manevich et al. 2004; Tew 2007; Townsend et al. 2009a).

S-glutathionylation is a specific post-translational modification of protein cysteine residues whereby glutathione may be reversibly bound to sulfhydryl groups (PSH) resulting in *S*-glutathionylated proteins (PSSG) (Townsend 2007; Dalle-Donne et al. 2008; Zhang et al. 2010). This process is promoted by oxidative/nitrosative stress but also occurs under non-stressed conditions and has a role in several physiological processes, namely glycolysis, calcium homeostasis, protein folding, cellular redox equilibrium and signal transduction (Klatt and Lamas 2000; Townsend 2007; Dalle-Donne et al. 2008).

Keap1 is an extremely thiol-rich protein, possessing a high number of cysteine residues some of which are predicted to be highly reactive (Dinkova-Kostova et al. 2002; Kensler et al. 2007; Rachakonda et al. 2008). Recently it was reported that upon depletion of GSH, cysteine residues in Keap1 are modified by *S*-glutathionylation and Nrf2 expression markedly increases (Zhang et al. 2010).

We previously demonstrated that GSTP expression is significantly increased in both *substantia nigra* (SN) and *striatum* (ST) of C57BL/6 mice brain after 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) single dose administration (Castro-Caldas et al. 2009b), and that GSTP knockout (GSTP ko) mice are more susceptible to MPTP neurotoxicity than wild-type mice (Castro-Caldas et al. 2012). Involvement of ubiquitin-proteasome system (UPS) impairment in neurodegeneration has been described in PD (McNaught and Jenner 2001; Olanow and McNaught 2006) and in PD experimental models (McNaught et al. 2004) along with the demonstration that proteasome inhibition may also induce the expression of neuroprotective factors (Inden et al. 2005; Yew et al. 2005). Since proteasome inhibitors are specific inducers of GSTP1 expression in rat liver epithelial cells (Usami et al. 2005), we hypothesized that GSTP expression may be modified by proteasome inhibition by a mechanism involving Nrf2 that could be important in the potential protective response to MPTP-induced oxidative stress. The consequent increase in GSTP expression would in turn potentiate Keap1 *S*-glutathionylation, leading to further disruption of the Keap1-Nrf2 complex and Nrf2 activation in a positive feedback regulatory loop, resulting in neuronal protection.

In this study we used C57BL/6 wild-type and GSTP ko mice treated with MPTP and MG132 to further elucidate the protective role of GSTP in mice brain. We demonstrate for the first time the *in vivo S*-glutathionylation of Keap1 and the consequent activation of the Nrf2 pathway, unraveling another molecular mechanism underlying GSTP elicited neuronal protection.

3.3 Materials and Methods

3.3.1 Animals and treatment

All procedures involving animals were carried out in accordance with the institutional, Portuguese and European guidelines (*Diário da República, 2.ª série N.º 121* of 27 June 2011; and 86/609/CEE, 2003/65/CE European Council Directives) and methods were approved by the Ethical Committee for Animal Experimentation of the Faculty of Pharmacy, University of Lisbon. Twelve week-old male C57BL/6 wild-type mice purchased from the Gulbenkian Institute of Science Animal House (Oeiras, Portugal) and C57BL/6 *Gstp1/p2* null mice lineage (Henderson et al. 1998b) re-derived and maintained at the same Animal House, were used. All animals were housed under standardized conditions on a 12 hours light-dark cycle, with constant temperature (22-24ºC) and humidity (50-60%) with free access to a standard diet and water *ad libitum*.

Mice were intra-peritoneally (i.p.) injected with either MPTP (Sigma, St Louis, MO, USA) at 40 mg/kg, or with MG132 (Boston Biochem, Cambridge, MA, USA) at 5mg/kg, or with both MPTP and MG132, at the same dosages. Control mice received saline alone. At six hours after MPTP, MG132 or vehicle administration, mice were anesthetized with sodium pentobarbital (50mg/kg, i.p.), decapitated and brains were dissected as previously described (Castro-Caldas et al. 2012).

3.3.2 Antibodies

The antibodies used in Western blot and Dot blot, Immunofluorescence and Co-Immunoprecipitation assays are listed in Table S1.

Table S1- List of the primary antibodies used in immunofluorescence, western blot and immunoprecipitation.

Protein	Reference	Source	Dilution	Application
GFAP	MAB360	Millipore, Temecula, CA, USA	1:500	IF*
Iba1	019-19741	Wako Chemicals, Germany	1:100	IF
Nrf2	ab31163	Abcam, Cambridge, UK	1:50	IF
GSTP	610719	BD Biosciences Pharmingen, San Diego, CA, USA	1:100	IF
			1:1000	IB§
Nrf2	MAB3925	R&D Systems, Minneapolis, MN, USA	1:500	IB
HO-1	SPA-896	Stressgen, Ann Arbor, MI, USA	1:1000	IB
Lamin B1	ab16048	Abcam, Cambridge, UK	1:5000	IB
Glutathione	SPA-542-E	Stressgen, Ann Arbor, MI, USA	1:1000	IB
			1μg	IP†
Keap1	#4617	Cell Signaling, Boston, MA, USA	1:1000	IB
DNP	D9656	Sigma, St Louis, MO, USA	1:2000	IB
β-actin	A5441	Sigma, St Louis, MO, USA	1:15000	IB

*IF=immunofluorescence §IB=immunoblot/western blot †IP=immunoprecipitation

3.3.3 Evaluation of reactive oxygen species

ROS levels were evaluated by fluorescence microscopy using the fluorescent probe 2',7'-dichlorofluorescein diacetate (DCF-DA; Molecular Probes, Invitrogen, Eugene, OR, USA). Mice were anesthetized and transcardially perfused with phosphate buffer saline (PBS). Brains were quickly removed and rinsed in 15% sucrose in PBS. Cryostat coronal sections (14μm thick) were permeabilized with 1% Triton X-100 in PBS for 20 min and incubated with 20 μM DCF-DA for 30 min at 37ºC. Sections were mounted with MOWIOL 4-88 reagent (Merck/Calbiochem, Darmstadt, Germany) and photographed under a Leica DM IRE2 microscope with an attached Leica DFC350 FX camera, (Leica Microsystems, Inc.).

3.3.4 Determination of protein carbonyls levels

Protein carbonyls levels were used as an indicator of protein oxidation and determined as previously described (Shang et al. 2001). Protein concentrations were adjusted to 1.5 mg/mL with lysis buffer (10 mM Tris-HCl pH7.6, 1% NP-40, 0.1% SDS, 5 mM EDTA) plus Complete Mini protease inhibitors cocktail (Roche Diagnostics,Penzberg, Germany). After sonication, samples were centrifuged at 15000*g*, at 4ºC, for 15 min. Proteins were derivatized using 2,4-dinitrophenylhydrazine (DNPH), pellets were solubilized in Laemmli buffer and dot blotted onto a nitrocellulose membrane. The membrane was probed with a rabbit anti-DNP antibody (Sigma). Levels of carbonyls were quantified by densitometric analysis.

3.3.5 Immunofluorescence

Mice were anesthetized and transcardially perfused with ice-cold PBS, followed by 4% paraformaldehyde in PBS. Brains were quickly removed, fixed in 2% paraformaldehyde/saturated picric acid for 24 hours and rinsed in 15% sucrose in PBS. Cryostat coronal brain sections (14μm thick) were permeabilized with 1% Triton X-100 in PBS, and blocked with goat serum (Sigma), incubated with primary antibodies overnight, at 4ºC, followed by incubation with Alexa Fluor 568 and 488 labeled secondary antibodies (Molecular Probes) and 1μg/ml of DAPI for nuclear staining, for 1 hour at room temperature. Sections were mounted with MOWIOL 4-88 reagent. Images were collected by confocal microscopy using a Carl Zeiss LSM 710 instrument (Carl Zeiss MicroImaging GmbH, Jena, Germany) and analyzed with Zen 2009 software (Carl Zeiss) and ImageJ (NIH, USA).

3.3.6 Assessment of glutathione levels

Glutathione levels were determined with a kinetic assay using the *Glutathione Assay Kit* (Sigma) according to manufacturer's instructions. After brain dissection, samples were immediately flash-frozen and grounded in a pestle and mortar with liquid nitrogen to prepare a fine powder. These samples were then deproteinized with 5% 5-sulfosalicylic acid solution, centrifuged at 10000*g*, at 4ºC, for 10 min. Supernatants were assayed for glutathione content and pellets used to estimate the protein content.

In this reaction, reduction of 5,5'-dithiobis(2-nitrobenzoic acid) to 5-thio-2-nitrobenzoic acid occurs and the oxidized glutathione (GSSG) formed is recycled by Glutathione Reductase (GR). The reaction rate is proportional to the concentration of glutathione in the sample and is measured by detection of the reaction product, at 412 nm. Concentrations of glutathione (nmol/mg tissue) were calculated from glutathione standard curve.

3.3.7 Determination of activities of glutathione related enzymes

Total GST enzymatic activity was evaluated using the *Glutathione S-Transferase Assay Kit* (Sigma) according to manufacturer's instructions. GSTs catalyze the conjugation reaction of glutathione with the 1-chloro-2,4-dinitrobenzene substrate. UV absorption rate at 340 nm is directly proportional to GST activity in the sample and was recorded as nmol/min/mg protein and plotted as percentage of control sample.

γ-Glutamyltranspeptidase (γ-GT) activity was determined according to the method described by Szasz (Szasz 1974) with modifications. In this reaction, formation of 5-amino-2-nitrobenzoate product that is proportional to γ-GT activity was measured at 405 nm.

Glutathione Peroxidase (GPx) activity was determined using the *Glutathione Peroxidase Activity Kit* (Enzo Life Sciences GmbH, Germany) according to manufacturer's instructions. Cumene hydroperoxide was used as GPx substrate and the decrease in NADPH absorbance at 340 nm was measured. For Glutathione Reductase activity determination *Glutathione Reductase Activity Kit* (Sigma) was used, following the manufacturer's instructions. The assay is based on the reduction of GSSG by NADPH in the presence of GR. The decrease rate in NADPH absorption at 340 nm is proportional to GR activity.

3.3.8 Western blot analysis

Tissue extracts from mouse midbrains and striata were prepared as previously described (Castro-Caldas et al. 2009b). Cytosolic and nuclear protein fractions were prepared as described by Levrand and collaborators with minor modifications (Levrand et al. 2005).

Samples were resolved by 10% or 12% SDS-PAGE and electroblotted onto Immobilon P (Millipore, Bedford, MA, USA). Membranes were incubated with primary antibodies overnight at 4ºC. The membranes were then incubated with horseradish peroxidase-conjugated anti-mouse (Cell Signaling, Boston, MA, USA) or anti-rabbit (Promega, Madison, WI, USA) secondary antibody, for 1 hour at room temperature. Proteins were detected by the ECL chemiluminiscent method and visualized with Hyperfilm ECL (GE Healthcare Biosciences, Uppsala, Sweden). Results were quantified using the Gel-Pro Analyzer densitometry analysis software (Media Cybernetics, MD, USA).

To investigate S-glutathionylation of the proteins, tissue extracts were separated under non-reducing conditions (without β-mercaptoethanol or dithiothreitol in loading buffer) and blots were probed with an anti-glutathione antibody.

3.3.9 Co-Immunoprecipitation assays

Co-immunoprecipitations were performed using aliquots (1mg) of total protein extracts from striata incubated with anti-glutathione antibody, at 4ºC, for 16 hours. Protein A/G plus agarose beads (20 μL; Santa Cruz Biotechnology, Santa Cruz, CA, USA) were added and incubated for 2 hours, at 4ºC. The immunocomplexes bound to the A/G agarose beads were washed 3 times with washing buffer (50 mM Tris-HCl pH 7.4, 180 mM NaCl, 1 mM EDTA, 1% Triton-X 100) re-suspended in Laemmli buffer and denatured at 100ºC for 5min. The Western blotting was performed as described above. Blots were probed with anti-Keap1 antibody.

3.3.10 Statistical analysis

Statistical analyses were performed using GraphPad/Prism 5.0 software (SanDiego, CA, USA). Analysis of variance (ANOVA) testing was performed using one-way analysis with Tukey's post-hoc test for multiple comparisons. Differences were considered statistically significant when $p<0.05$.

3.4 Results

3.4.1 Increased ROS production and protein carbonyls formation upon MPTP-induced neurotoxicity and proteasome inhibition

The systemic administration of MPTP, a widely used experimental animal model of parkinsonism (Dauer and Przedborski 2003), is known to involve mitochondrial complex I activity impairment, leading to ATP depletion, increased production of ROS and changes in cellular redox status (Jenner 2003; Przedborski et al. 2004). Therefore, we evaluated the deleterious effects of redox imbalance using this experimental paradigm (Saporito et al. 1999; Castro-Caldas et al. 2009b) and investigated the effects of proteasomal impairment on ROS formation and protein oxidative modifications. ROS levels were evaluated on striatal coronal sections of wild-type mice brain using the fluorescent probe DCF-DA. Photomicrographs show significantly increased intracellular ROS formation after MPTP treatment ($p<0.01$; Figure 3.1A and B). Moreover, the concomitant administration of MPTP and MG132 led to a synergistic increase in ROS production when compared to MPTP treated animals ($p<0.001$). In agreement, an increase in protein carbonyls accumulation, indicative of the presence of oxidized proteins, was also observed in mouse *striatum* in response to MG132, MPTP and MG132 plus MPTP (Figure 3.1C).

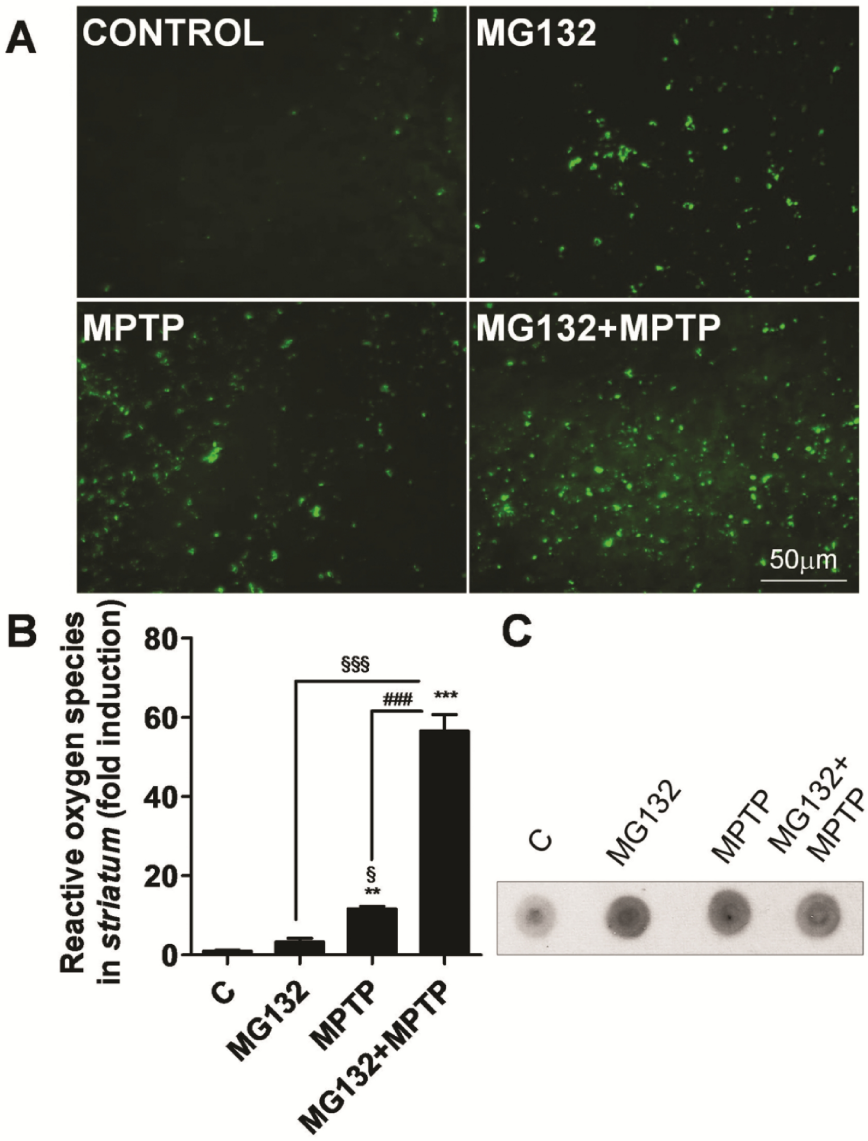

Figure. 3.1. Reactive oxygen species production and protein oxidation in mice *striatum* following MPTP and MG132 administration. Six hours after administration of MG132 (5 mg/Kg) and/or MPTP (40 mg/Kg), C57BL/6 wild-type mice were sacrificed and brains dissected. (**A**) ROS levels were evaluated on coronal brain sections at the level of *striatum* (Bregma 1.00) using the fluorescent probe DCF-DA. Fluorescence microscope images from saline (control), MG132, MPTP, and MG132 plus MPTP-treated mice are representative of three independent experiments. **(B)** Fluorescence intensity of randomly selected fields was quantified using Image J software. Data are presented as mean fluorescence units ± SEM expressed as fold induction relative to control. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test, where $^{**}p<0.01$, $^{***}p<0.001$ relative to control, $^{\S}p<0.05$, $^{\S\S\S}p<0.001$, relative to MG132 and $^{\#\#\#}p<0.001$ relative to MPTP. **(C)** Protein-carbonyls levels, indicative of protein oxidation, were detected by dot-blot analysis with anti-DNP antibody after DNPH-derivatization of tissue extracts from MG132, MPTP or MG132 plus MPTP treated mice. *Scale bar* = 50 µm

3.4.2 MPTP and MG132 induce astrogliosis and microgliosis in mice midbrain and *striatum*

We looked at astrogliosis and microgliosis in wild-type mouse brain by immunostaining with anti-GFAP and anti-Iba-1 antibodies, astrocyte- and microglia-specific markers, respectively. In coronal sections corresponding to both SN and ST we could observe features of reactive astrocytes (Figure 3.2) and microglia activation (Figure 3.3) upon treatment with MPTP or with MG132. Nevertheless, in MG132-treated mice, the inflammatory response occurred with a higher magnitude. The administration of both compounds together had a synergistic effect, revealing enhanced astrogliosis and microgliosis in both brain regions.

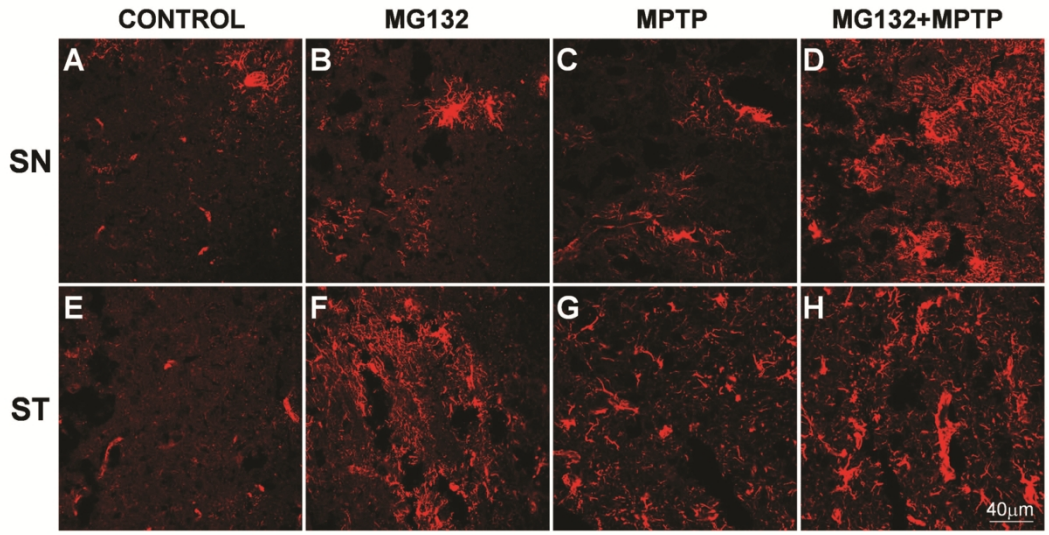

Figure 3.2. Astrocytes reactivity in mice *substantia nigra* and *striatum* in response to administration of MPTP and MG132. C57BL/6 wild-type mice were i.p. injected with MPTP (40 mg/Kg) and/or MG132 (5 mg/Kg) and sacrificed six hours post-treatment. Astrocytes were identified by glial fibrillary acidic protein (GFAP) immunostaining in coronal sections at the level of SN (Brema -3.20) (A-D) or at the level of ST (Brema 1.00) (E-H) from control (**A** and **E**) MG132 (**B** and **F**), MPTP (**C** and **G**) or MG132 plus MPTP (**D** and **H**) treated mice. All confocal immunofluorescence images were taken using the same laser power, gain and objective. Photomicrographs shown are representative of three independent experiments with two animals per group. *Scale bar* = 40 µm

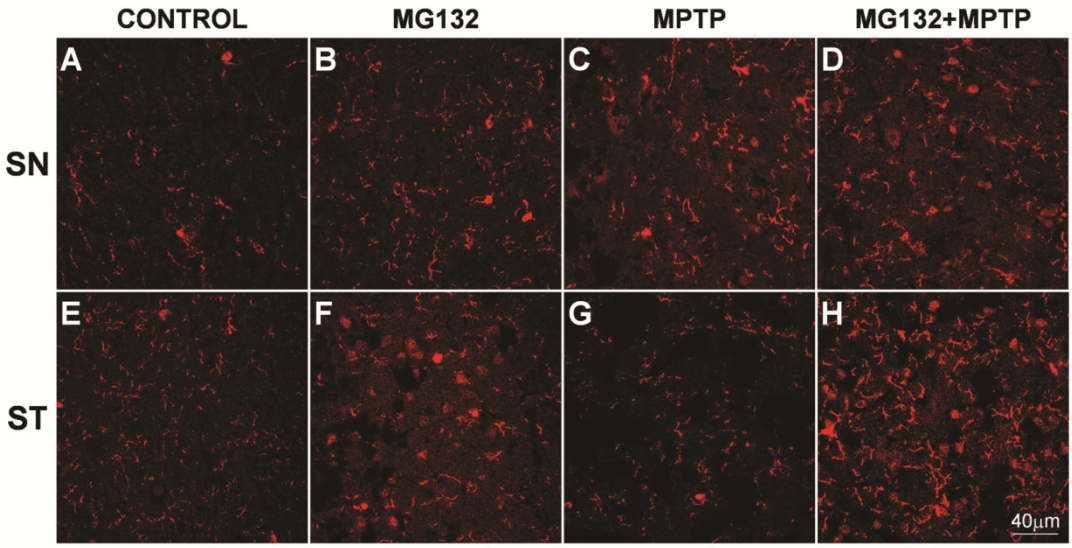

Figure. 3.3. Microgliosis in mice *substantia nigra* and *striatum* after MPTP and MG132 administration. C57BL/6 wild-type mice were i.p. injected with MPTP (40 mg/Kg) and/or MG132 (5 mg/Kg) and sacrificed six hours post-treatment. Microglia was identified by Iba-1 immunostaining in coronal sections at the level of SN (Brema -3.20) (A-D) or at the level of ST (Brema 1.00) (E-H) from control (**A** and **E**) MG132 (**B** and **F**), MPTP (**C** and **G**) or MG132 plus MPTP (**D** and **H**) treated mice. All confocal immunofluorescence images were taken using the same laser power, gain and objective. Photomicrographs shown are representative of three independent experiments with two animals per group. *Scale bar* = 40 µm

3.4.3 MPTP and MG132 decrease glutathione levels and induce alterations in glutathione metabolism

A marked depletion of both total (GSx) and reduced (GSH) glutathione levels is one of the earliest biochemical changes seen in PD (Sian et al. 1994b). In order to determine the effect of MPTP or MG132 treatments on the cellular redox state we measured both total and oxidized (GSSG) levels of glutathione, in mouse midbrain and *striatum*. Although exposure to either MPTP or MG132 did not alter the GSx content in the midbrain (Figure 3.4A), at least at the time points and dosage used, an early and significant decrease in the GSx content in the *striatum* was observed (Figure 3.4B). This imbalance might be due to a decrease in GSH levels, although we did not detect any concomitant increase in GSSG content. Surprisingly we did not observe significant changes in GSx levels in the midbrain and *striatum* of mice treated with both MG132 and MPTP.

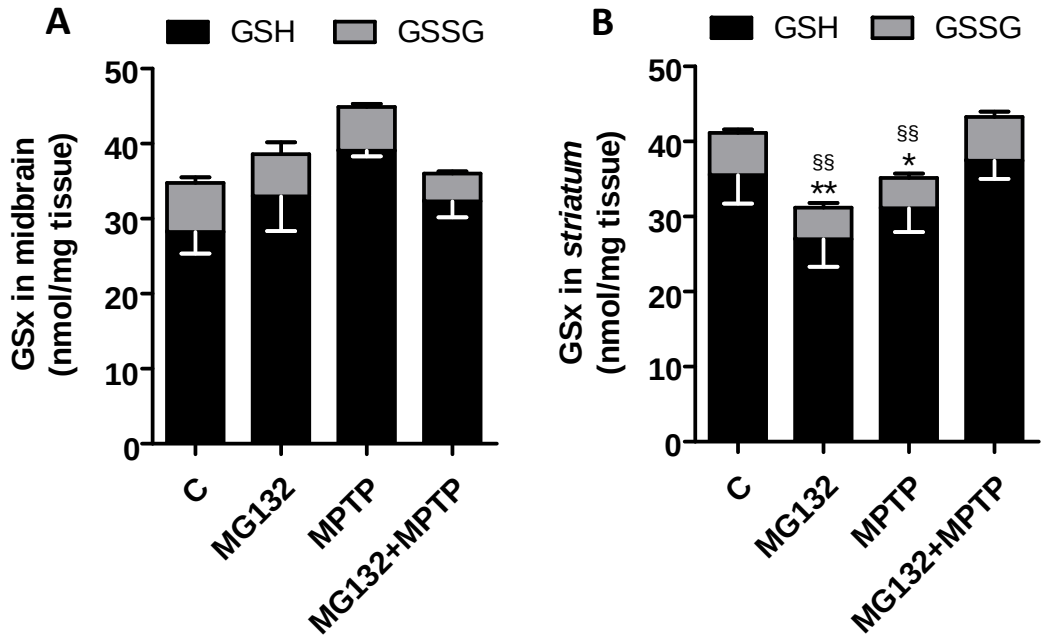

Figure 3.4. Effect of MPTP and proteasome inhibition on total free glutathione levels in mice midbrain and *striatum*. C57BL/6 mice were i.p. injected with saline (control), MG132 (5 mg/Kg) and/or MPTP (40 mg/Kg), sacrificed after six hours and brains were dissected. Glutathione content in mice midbrains and *striata* was assessed after deproteinization of the samples. Concentrations of glutathione were calculated from standard curve and expressed as nmol/mg tissue. Total free glutathione levels (GSx) as well as the content in reduced (GSH) and oxidized (GSSG) glutathione in mice midbrain **(A)** and *striatum* **(B)** are presented. Data are expressed as mean ± SEM of at least three independent experiments. Statistical analysis of GSx was carried out using one-way ANOVA with Tukey post-hoc test, where $^{*}p<0.05$, $^{**}p<0.01$, relative to control, $^{§§}p<0.01$ relative to MG132 plus MPTP. *Grey bars*: GSSG, *Black bars*: GSH.

Next, we measured the activity of a range of enzymes involved in glutathione-dependent processes to assess if the reduction in GSx concentration might be associated with altered catalytic activity of any of the different players in glutathione metabolism.

As shown in Figure 3.5A, the catalytic activity of GST is unchanged in MPTP and/or MG132 treated mice, indicating that the observed lowered levels of total free glutathione are not due to altered enzymatic activity in the conjugation reactions mediated by GST. Depletion of GSH may also be explained by an increased efflux, promoted by γ-GT. However, the observation that γ-GT levels are significantly decreased by either MPTP ($p<0.05$) or MG132 ($p<0.01$) administration (Figure 3.5B) suggests that the lowering of GSH content is probably not due to its export out of cells.

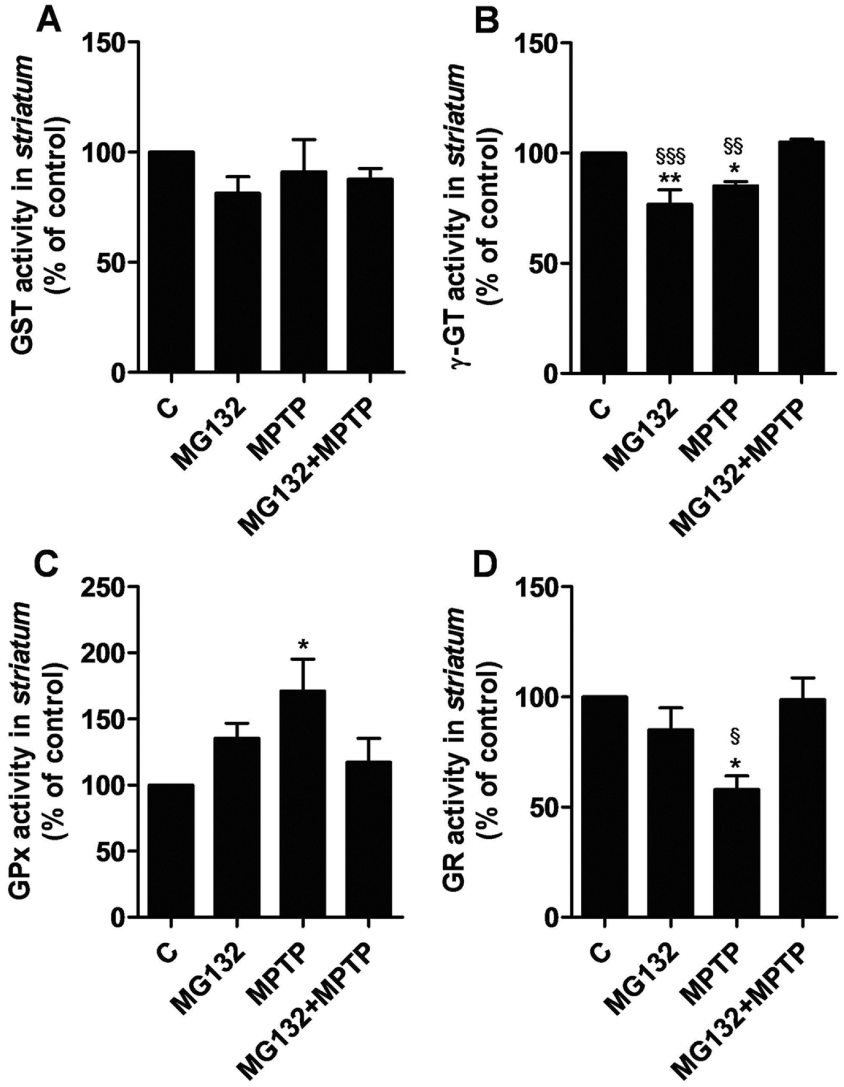

Figure 3.5. Glutathione related enzymes catalytic activities in mice *striatum* after MPTP and MG132 administration. Tissue extracts were prepared from *striata* of C57BL/6 wild-type control mice (saline) or i.p. injected with, MPTP (40 mg/Kg), MG132 (5 mg/Kg) or with both compounds and sacrificed six hours post-treatment. Specific activities of GSTs **(A),** γ-GT (**B**) GPx (**C**) and GR (**D**) were determined using standard enzymatic assays. Data shown are mean ± SEM of three independent experiments and are depicted as percentage of control. Statistical analysis was carried out using one-way ANOVA with Tukey post-hoc test, where $^{*}p<0.05$, $^{**}p<0.01$, relative to control and $^{\S}p<0.05$, $^{\S\S}p<0.01$, $^{\S\S\S}p<0.001$ relative to MG132 plus MPTP.

GSH may also directly react with free radicals leading to its non-enzymatic inactivation, or be an electron donor in the reduction of peroxides catalyzed by GPx. Since GSH is oxidized to GSSG by GPx and reduced back to GSH by action of GR, we also evaluated the activity of these enzymes. Interestingly, GPx activity was significantly

increased ($p<0.05$) (Figure 3.5C) and GR activity was significantly decreased ($p<0.05$) (Figure 3.5D) upon treatment with MPTP. However, the activity of all of the analyzed glutathione-related enzymes was not significantly altered in the *striatum* of mice submitted to both MPTP and MG132 injections (Figure 3.5).

3.4.4 Activation of Nrf2 pathway through Keap1 *S*-glutathionylation

One other important biological function of glutathione is the maintenance of the redox status of protein sulfydryls through the formation of protein-glutathione-mixed disulfides, PSSG. In order to evaluate if the observed changes in glutathione levels and glutathione oxidation status may be reflected in alterations of protein *S*-glutathionylation pattern in mice brain, non-reducing Western blot analysis was done using a specific monoclonal anti-glutathione antibody that recognizes *S*-glutathionylated proteins. Tissue extracts from both wild-type and GSTP ko mice were used to assess if GSTP may play a role in protein *S*-glutathionylation under MPTP and/or MG132 insults.

As shown in Figure 3.6A, a different pattern of *S*-glutathionylation was observed when comparing wild-type to GSTP ko mice brain samples. Indeed, increased levels of *S*-glutathionylated proteins were detected in the striatum of both MPTP and/or MG132 treated wild-type mice when compared to the corresponding controls. Moreover, an even higher amount of *S*-glutathionylated proteins was detected in mice injected with both compounds. Interestingly, in the GSTP ko mice the striatal *S*-glutathionylated proteins detected in control mice were higher than the basal levels observed in the counterpart wild-type, but these levels were not altered by either MPTP or MG132 treatments.

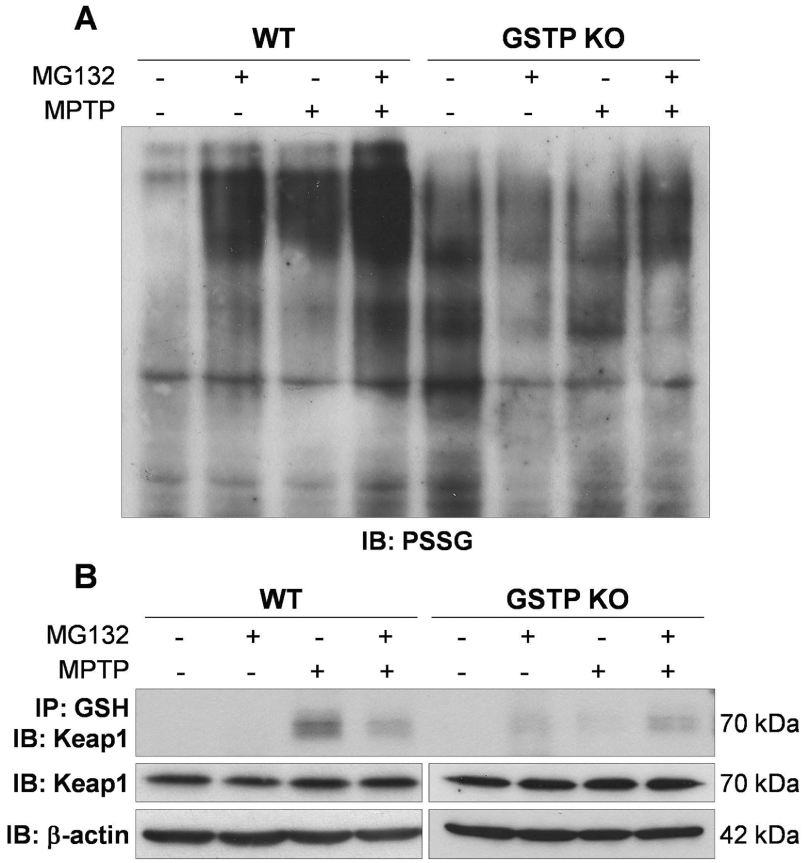

Figure 3.6. Protein *S*-glutathionylation in wild-type and GSTP ko mice. Keap1 *S*-glutathionylation in response to MPTP administration. Tissue extracts were prepared from *striata* of saline (control), MPTP (40 mg/Kg) and/or MG132 (5mg/Kg) treated C57BL/6 wild-type and GSTP ko mice. **(A)** Samples were separated under non-reducing conditions by SDS-PAGE and *S*-glutathionylated proteins (PSSG) detected by immunoblotting (IB) with anti-glutathione monoclonal antibody**. (B)** Keap1 *S*-glutathionylation was evaluated by co-immunoprecipitation (IP) with an anti-glutathione antibody followed by immunoblot analysis (IB) using an anti-Keap1 antibody. The immunoblots presented are representative of three independent experiments.

Next, by performing co-immunoprecipitation assays we were able to show that MPTP-induced oxidative stress led to a specific increase in the levels of *S*-glutathionylated Keap1 in the wild-type mice, that is not evident in the GSTP ko mice (Figure 3.6B). The specific modification of Keap1 cysteine residues might disrupt the Nrf2-Keap1 complex leading to Nrf2 nuclear accumulation and subsequent activation of target genes expression. Thus, we evaluated striatal Nrf2 total protein levels, Nrf2 nuclear translocation and the expression of Nrf2 downstream targets. Significantly higher levels of Nrf2 protein were detected in striatal extracts from MPTP-treated mice (Figure 3.7A

and B). Assessment of Nrf2 translocation by immunohistochemistry and Western blot analysis show that treatment with MG132 or MPTP induced Nrf2 translocation to the nucleus (Figure 3.7C, D and E).

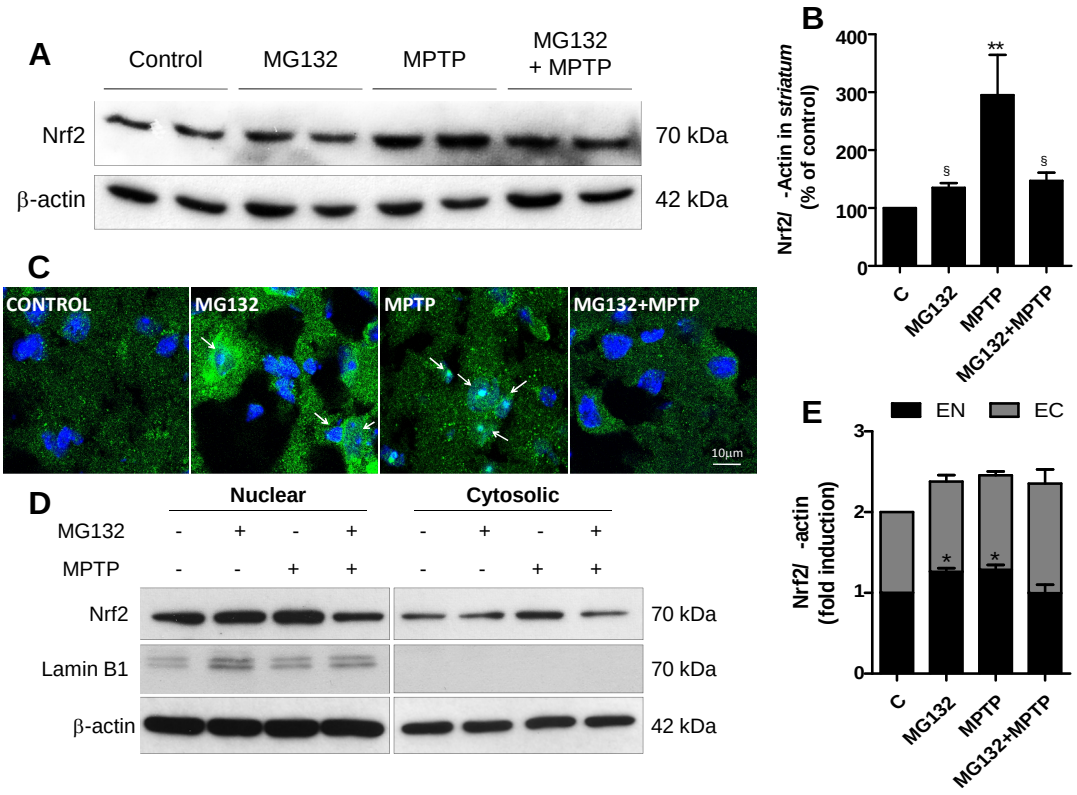

Figure 3.7. Nrf2 expression and sub-cellular localization following MPTP or MG132 administration. C57BL/6 wild-type mice were i.p. injected with MPTP (40 mg/Kg) and/or MG132 (5 mg/Kg) sacrificed six hours post-treatment and striata were dissected. **(A, B)** Tissue extracts were subjected to SDS-PAGE and the corresponding blots were probed with a mouse anti-Nrf2 antibody. Analysis of β-actin was done in parallel as a loading control. The immunoblots presented are representative of three independent experiments and show the results obtained with duplicate samples *per* experimental condition. The relative levels of Nrf2 in saline-treated control samples was arbitrarily set as 100% and its levels in MPTP and/or MG132-treated samples were calculated and plotted as a percentage of this value. Data shown are mean ± SEM of at least three independent experiments ($^{**}p<0.01$ relative to control, $^{\S}p<0.05$ relative to MPTP; ANOVA with Tukey post-hoc test). **(C)** Coronal mice brain sections at the level of *striatum* (Bregma 1.00) were immunostained with a rabbit anti-Nrf2 antibody (green). Dapi (blue) was used as nuclear marker. *Arrows* indicate nuclear localization. *Scale bar* = 10µm. **(D** and **E)** Nrf2 nuclear translocation was analyzed in nuclear (EN) and cytosolic (EC) extracts. Samples were subjected to SDS-PAGE and the corresponding blots were probed with a mouse anti-Nrf2 antibody. Analysis of Lamin B1 was done in parallel as a control for the nuclear protein extracts and β-actin was used as a loading control. Data shown are mean ± SEM of at least three independent experiments and are expressed as fold induction relative to control. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test, where $^{*}p<0.05$ relative to control.

In order to evaluate if the Nrf2 pathway was activated we analyzed the protein expression levels of the Nrf2 downstream targets, GSTP and heme oxygenase (HO-1). Results presented in Figure 3.8 show a significant increase of GSTP and HO-1 protein levels in the *striatum* after acute MPTP administration to wild-type mice (Figure 3.8A, B and C). Conversely, in GSTP ko mice no changes in HO-1 protein levels were detected (Figure 3.8D). Moreover, by confocal immunofluorescence we could detect the co-localization of Nrf2 protein with GSTP in the *striatum* (Figure 3.8E).

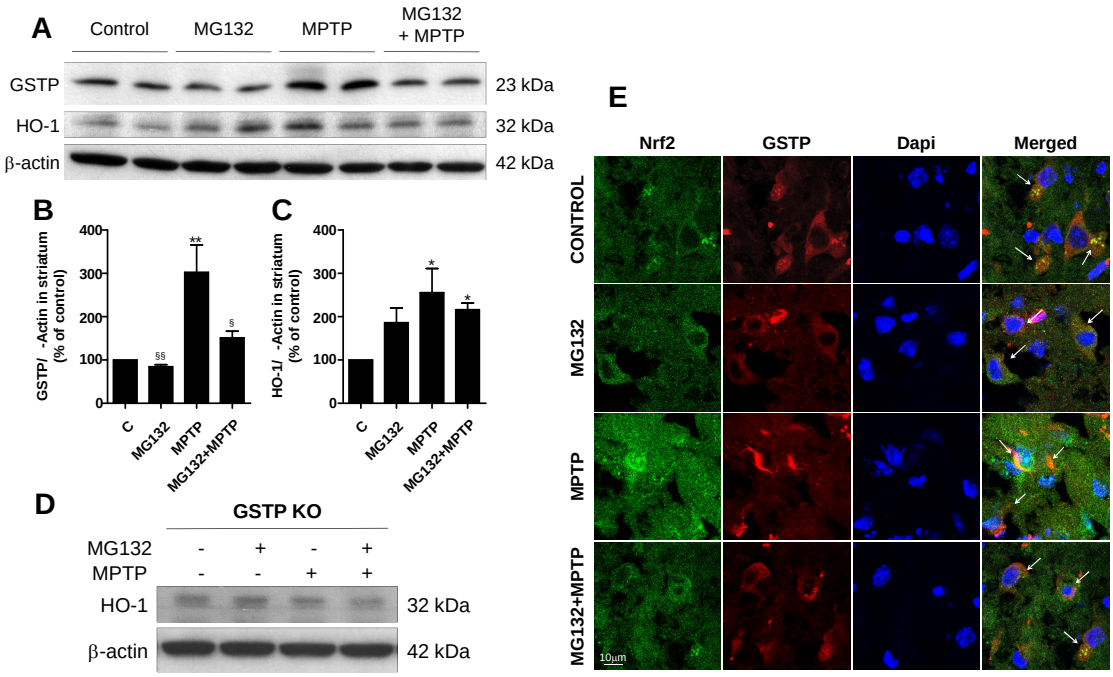

Figure 3.8. GSTP and HO-1 expression levels in response to MPTP and MG132 administration. C57BL/6 wild-type mice were i.p. injected with either MPTP (40 mg/kg) or MG132 (5mg/kg) or with both compounds. Controls were i.p. injected with saline. Mice were sacrificed six hours post-injection. **(A)** GSTP and HO-1 expression levels in tissue extracts obtained from dissected *striata* of wild-type mice were assessed by Western blot analysis. The immunoblots presented are representative of three independent experiments and show the results obtained with duplicate samples *per* experimental condition. Analysis of β-actin was done in parallel as a loading control. **(B)** and HO-1 **(C)** in wild-type mice saline-treated control samples were arbitrarily set as 100% and its levels in MPTP and/or MG132-treated samples was calculated and plotted as a percentage of this value. Data shown are mean ± SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test where $^{*}p<0.05$, $^{**}p<0.01$, relative to control, $^{\S}p<0.05$, $^{\S\S}p<0.01$ relative to MPTP. **(D)** HO-1 content in GSTP ko mice striata was also analyzed by Western blot. The relative levels of GSTP **(E)** Coronal brain sections at the level of *striatum* (Bregma 1.00) from C57BL/6 wild-type mice were double immune stained for Nrf2 protein (green) and GSTP (red). Dapi (blue) was used as nuclear marker. Representative confocal microscopy images from control (C), MG132 and MPTP treated mice of three independent experiments are shown. *Arrows* indicate co-localization. *Scale bar* = 10μm

3.5 Discussion

In the present study we used MPTP administration to mice as an experimental model of PD (Dauer and Przedborski 2003) along with MG132, as proteasome inhibition has been suggested to recapitulate some of the pathological features of nigral cells degeneration (McNaught et al. 2004), to assess early events that trigger molecular mechanisms of antioxidant defense.

In accordance with the previously described mechanism of MPTP selective dopaminergic neurotoxicity (Przedborski et al. 2004), we observed increased ROS production and accumulation of protein carbonyls in the *striata* of mice upon MPTP administration that were potentiated by the concomitant proteasome inhibition, expanding the knowledge of the *in vivo* molecular mechanisms triggered by MPTP-induced oxidative stress when the UPS is impaired.

One of the hallmarks of the inflammatory response triggered by MPTP is the increased number of activated astrocytes and microglia in the nigrostriatal region (Jazwa et al. 2011). Accordingly, increased astrogliosis and microgliosis were detected upon treatment with MPTP, and this response was also exacerbated by the simultaneous administration of MG132, and was more pronounced in the *striatum.* This is in agreement with previous reports, suggesting that striatal dopaminergic nerve terminals are the primary target of the degenerative process in PD and that neuronal death may result from a “dying-back process” (Jackson-Lewis et al. 1995).

Also in the *striatum*, total glutathione levels were found to be significantly decreased in both MPTP and MG132-treated mice. Nevertheless, concomitant changes in the activity of enzymes involved in glutathione homeostasis were more evident in MPTP-treated mice. Conjugation of GSH with electrophilic substrates, catalyzed by GST is a mechanism of cellular protection (Ketterer 2001) but also a “glutathione-consuming” process. In this study, no significant changes in the catalytic activity of GST were detected that might account for the observed decrease in glutathione levels. Detoxification of ROS mediated by GSH, both non-enzymatic and enzymatic (*via* GPx) leads to the production of GSSG. Due to its toxicity, GSSG must be re-converted to GSH, conjugated or transported out of cells (Ghezzi et al. 2005; Maher 2006). The observed significant increase in GPx activity may be due to a cellular response to the increased production of peroxides triggered by MPTP and leads to increased conversion of GSH to GSSG.

However, we also observe a decrease in GR activity, which implies that GSH is not being regenerated at a normal rate. In fact, GR is itself an enzyme susceptible to oxidative damage and a decrease in its activity was reported in brain upon glutathione depletion (Barker et al. 1996). Similar to what we observed in the *striatum* of MPTP-treated mice, Marsden and collaborators described a decrease in GSH that was not accompanied by a corresponding increase in GSSG levels in PD *post mortem* brains (Sian et al. 1994b; Sian et al. 1994a). The absence of an increase in GSSG levels does not seem to be due to an increased of export through γ-GT, since we also detected a decrease in the activity of this enzyme. Since neurons rely on astrocytes for supplying precursor amino acids required for GSH synthesis (Dringen and Hirrlinger 2003), the decrease in the observed γ-GT activity might have as a consequence a decrease in the release of amino acids from astrocytes, thus limiting GSH synthesis in neurons.

Another mechanism for the cell to deal temporarily with excess of GSSG is *S*-glutathionylation, particularly by thiol-disulfide exchange (Ghezzi 2005). *S*-glutathionylation temporarily protects protein cysteine residues from irreversible oxidation, since this post-translational modification is reversible, by action of glutaredoxin (Klatt and Lamas 2000; Maher 2006). Also, it allows the maintenance of the glutathione pool that otherwise, in the form of GSSG, would be exported from the cell (Ghezzi et al. 2005; Maher 2006). Thus, the decreased levels of total free glutathione may result from its conjugation with proteins through *S*-glutathionylation.

Nrf2 has been described to protect against neurotoxic insults and Nrf2 null mice display increased sensitivity to MPTP (Burton et al. 2006; Chen et al. 2009; Jazwa et al. 2011). Nrf2 activity is mainly regulated by Keap1 (Kobayashi et al. 2004; Kobayashi et al. 2006; Nguyen et al. 2009), which possesses a high number of reactive cysteine residues (Dinkova-Kostova et al. 2002; Maher 2006; Kensler et al. 2007). Modifications of these residues by oxidative stress may lead to disruption of the Nrf2-Keap1 complex, thereby decreasing Nrf2 ubiquitination and proteasomal degradation (Kobayashi et al. 2006). Our results reveal that Keap1 is *S*-glutathionylated *in vivo* in MPTP-treated wild-type mouse brain and that this post-translational modification most likely disrupts the Nrf2-Keap1 complex, consequently leading to induced expression of Nrf2 downstream targets, HO-1 and GSTP. Corroborating our data, Ma and collaborators have recently reported that pharmacological-induced depletion of glutathione activates Nrf2 through Keap1 *S*-glutathionylation in hepatoma cells (Zhang et al. 2010). Our results show

specific Keap1 *S*-glutathionylation in MPTP-treated wild-type mice striata, a feature which is not observed in the GSTP ko mice. In accordance with this, HO-1 protein expression remained unchanged in the striata of these mice. Since both the pattern of global *S*-glutathionylation and specific Keap1 *S*-glutathionylation differ between wild-type and GSTP ko mice, our results suggest an important role for GSTP in the *in vivo* control of protein *S*-glutathionylated levels.

Taken as a whole, our results suggest that MPTP triggers a decrease in GSH that is not accompanied by a corresponding increase in GSSG levels, due to an overall increase in *S*-glutathionylation. Specifically, the increase in GSTP-mediated Keap1 *S*-glutathionylation *in vivo* in mice brain is a possible *feedback* regulatory mechanism that promotes the activation of Nrf2.

Overall the results presented herein demonstrate that MPTP induces early significant changes in the redox status in the mouse brain. In response to this insult the main cellular antioxidant mechanisms, namely those regulated by Nrf2, are triggered. However, when the UPS is impaired cells are no longer able to mount an adequate antioxidant protective response against the MPTP insult. Our results further strengthen the hypothesis of the impairment of protein degradation pathways as a contributing factor in the neurodegenerative processes and unravel an additional molecular mechanism contributing to GSTP-elicited neuronal protection.

Acknowledgments

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4

UBIQUITIN-PROTEASOME SYSTEM IMPAIRMENT AND MPTP-INDUCED OXIDATIVE STRESS IN THE BRAIN OF C57BL/6 WILD-TYPE AND GSTP KNOCKOUT MICE

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4.1 Abstract

The ubiquitin-proteasome system (UPS) is the primary proteolytic complex responsible for the elimination of damaged and misfolded intracellular proteins, often formed upon oxidative stress.

Parkinson's disease (PD) is neuropathologically characterized by selective death of dopaminergic neurons in the *substantia nigra* (SN) and by the accumulation of intracytoplasmic inclusions of aggregated proteins. Along with mitochondrial dysfunction and oxidative stress, defects in the UPS with failure to degrade misfolded and aggregated proteins have been implicated in PD.

Glutathione S-Transferase pi (GSTP) is a phase II detoxifying enzyme that has been proven to play an important defensive role against the accumulation of reactive metabolites that potentiate the aggression of SN neuronal cells, by regulating several processes including *S*-glutathionylation, modulation of glutathione levels and control of kinase-catalytic activities.

In this work we used C57BL/6 wild-type and GSTP knockout mice treated with MPTP and a proteasome inhibitor (MG132) to investigate the modulation of the UPS function in the midbrain and striatum.

The results presented here demonstrate that different components of the UPS have different susceptibilities to oxidative stress. Importantly, when compared to the wild-type, GSTP knockout mice display decreased ubiquitination capacity and overall increased susceptibility to UPS damage and inactivation upon MPTP-induced oxidative stress.

4.2 Introduction

The ubiquitin-proteasome system (UPS) is the major non-lysosomal protein degradation pathway within cells (Ciechanover and Brundin 2003; Schwartz and Ciechanover 2009). Besides being the primary route for degradation of misfolded intracellular proteins, through the regulation of protein turnover, the UPS is critical for numerous cellular functions including regulation of cell cycle and division, survival and cellular response to stress, apoptosis and intracellular signaling (Ciechanover and Brundin 2003; Schwartz and Ciechanover 2009).

The degradation of proteins by the UPS is a sequential process involving an initial step of ubiquitin conjugation to the protein substrate followed by the degradation of the tagged protein through the 26S proteasome complex with release of free ubiquitin (Glickman and Ciechanover 2002; Ciechanover and Brundin 2003). In this pathway the ubiquitin-activating enzyme (E1) activates ubiquitin through the formation of a high-energy thiol ester bond between the C-terminal glycine residue of ubiquitin and a specific cysteine in the active site of the E1 enzyme, in an ATP-dependent reaction (Haas and Rose 1982; Haas et al. 1982; Glickman and Ciechanover 2002). Subsequently, ubiquitin is transferred to one of several ubiquitin-conjugating enzymes (E2) to which ubiquitin is also linked *via* a thiol ester bond (Pickart and Rose 1985; Haas and Bright 1988). Some E2 enzymes catalyze the covalent attachment of ubiquitin to a lysine residue in the substrate proteins directly, whereas other E2 enzymes act in concert with ubiquitin protein ligases (E3) (Hershko et al. 1983). The combination of E2s and E3s determines the substrate specificity. The ubiquitinated proteins can then be recognized and degraded by the proteasome.

Many neurodegenerative diseases are characterized by accumulation of misfolded protein deposits in affected brain regions, suggesting a failure in the cellular protein degradation pathways (Taylor et al. 2002). Parkinson's disease (PD) is neuropathologically characterized by selective death of dopaminergic neurons in the *substantia nigra* (SN) and by the accumulation of intracytoplasmic inclusions of aggregated proteins (Dauer and Przedborski 2003).

Although the molecular mechanisms underlying neurodegeneration in sporadic PD are still unclear, solid evidence has accumulated implicating mitochondrial dysfunction, oxidative stress and neuro-inflammation in the pathogenesis of the disease

(Moore et al. 2005). Moreover, defects in the UPS with failure to degrade misfolded and aggregated proteins have been implicated in both familial and sporadic forms of PD (McNaught and Jenner 2001; Dawson and Dawson 2003; Olanow and McNaught 2006; McNaught et al. 2010).

Evidences for UPS dysfunction in PD include the mutations in genes associated with protein processing and degradation, namely parkin (Kitada et al. 1998) and ubiquitin C-terminal hydrolase-L1 (Leroy et al. 1998) found in patients with familial PD (reviewed by Dawson and Dawson (Dawson and Dawson 2003)). Moreover, impairment of UPS has been reported in sporadic PD, with patients displaying impaired proteasomal activity in the SN (McNaught and Jenner 2001) but not in the *striatum* (ST) (Furukawa et al. 2002). Evidences for the involvement of UPS impairment in neurodegeneration has also been described in PD experimental models (McNaught et al. 2004).

Glutathione S-transferase pi (GSTP) belongs to a family of phase II drug metabolizing enzymes that catalyze the detoxification of electrophiles by conjugation with glutathione (Henderson et al. 1998a; Hayes et al. 2005; Henderson and Wolf 2011). In previous studies we showed that GSTP expression is significantly increased in both SN and ST of C57BL/6 mice after a single MPTP injection (Castro-Caldas et al. 2009b) and that GSTP knockout (GSTP ko) mice are more susceptible to MPTP neurotoxicity than wild-type mice (Castro-Caldas et al. 2012). Furthermore, we (Castro-Caldas et al. 2012) and others (Adler et al. 1999; Wang et al. 2001; Smeyne et al. 2007) demonstrated that GSTP protects against MPTP-induced toxicity by direct modulation of c-Jun N-terminal kinase (JNK) activity (Adler et al. 1999; Wang et al. 2001; Castro-Caldas et al. 2012) and by modulation of the nuclear factor-erythroid 2-related factor2 (Nrf2) pathway through *S*-glutathionylation of Keap1 (Neves Carvalho *et al* unpublished results, see chapter 3 of this thesis).

Removal of oxidative-damaged proteins by the UPS is essential for the cells to cope with environmental stresses (Jahngen-Hodge et al. 1997; Dudek et al. 2005). As the UPS is known to prefer oxidized proteins to their native forms as substrates this is an important protein quality control mechanism. However, oxidation can impair the components of the UPS both at the level of ubiquitination and of proteasomal degradation, defining the UPS itself as a target for oxidative stress.

Since UPS impairment has been suggested to participate in the process of neurodegeneration in PD, with the present study we aim to further characterize the extent

and mechanisms of UPS impairment in the well established MPTP experimental model of PD. Moreover, we assessed whether the absence of GSTP could be a determinant factor in the failure of the UPS under oxidative stress situations.

The results present herein show that in mice brain, different components of the UPS display different susceptibilities to MPTP-induced oxidative stress and to proteasome inhibition. Importantly, upon MPTP and MG132 insults GSTP ko mice are more susceptible to UPS failure indicating that GSTP has also a role in protecting protein degradation pathways against oxidative stress.

4.3 Materials and Methods

4.3.1 Materials

MPTP was purchased from Sigma (St Louis, MO, USA). $Na^{125}I$ was purchased from Perkin Elmer (Boston, MA, USA). Proteasome inhibitor (MG132) and the fluorogenic peptide substrate Succcinyl-Leucine-Leucine-Valine-Tyrosine-7-amido-4-methylcoumarin (Suc-LLVY-AMC) were purchased from Boston Biochem (Cambridge, MA, USA) and Centricon-10 microconcentrators were from Millipore Corporation (Bedford, MA, USA). Coomassie Plus Protein assay and BCA reagents were obtained from Pierce (IL, USA), and Complete Mini protease inhibitors cocktail from Roche Diagnostics (Penzberg, Germany). Immobilon P membrane was from Millipore and ECL and Hyperfilm ECL were purchased from GE Healthcare Biosciences (Uppsala, Sweden). The following antibodies were used: mouse anti-ubiquitin clone P4D1 (Covance, Princeton, NJ, USA), rabbit anti-E1 (provided by Dr. Fu Shang from Tufts University, Boston, MA, USA) and mouse anti-β-actin (Sigma) and horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit (BioRad, Hercules, CA, USA). Unless otherwise specified, all other reagents were of the highest analytical grade and were purchased from Sigma.

4.3.2 Animals and treatment

Experiments involving the use of animals were carried out in accordance with the institutional, Portuguese and European guidelines (*Diário da República, 2.ª série N.º 121* of 27 June 2011; and 86/609/CEE, 2003/65/CE European Council Directives) and methods were approved by the Ethical Committee for Animal Experimentation of the Faculty of Pharmacy, University of Lisbon. Twelve week-old male C57BL/6 wild-type mice purchased from the Gulbenkian Institute of Science Animal House (Oeiras, Portugal) and C57BL/6 *Gstp1/p2* null mice lineage (Cancer Research UK; (Henderson et al. 1998b)) re-derived and maintained at the same Animal House, were used. All animals were housed under standardized conditions on a 12 hours light-dark cycle, with constant temperature (22-24ºC) and humidity (50-60%) with free access to a standard diet and water *ad libitum*.

Mice were intra-peritoneally (i.p.) injected with either MPTP at 40 mg/kg, MG132 at 5mg/kg, or with both MPTP and MG132, at the previous indicated dosages. Control mice received saline alone. At 6 or 24 h after MPTP, MG132 or vehicle administration mice were sacrificed under anesthesia with sodium pentobarbital (50 mg/kg, i.p.) and brain dissected as previously described (Castro-Caldas et al. 2009b). Groups of at least three mice were used for each condition (time point/dosage).

4.3.3 Proteasome Activity

Mice midbrain and *striatum* were dissected and homogenized in 50 mM Tris-HCl pH 7.6. The homogenate was then centrifuged at 15000*g*, at 4ºC, for 15 min. The protein concentration was assayed from the resulting supernatants with BCA Protein assay reagents. The mixture, containing 20 μg of tissue extract, was incubated at 37°C for 1 h with 70 μM of peptide substrate Suc-LLVY-AMC to assess the chymotrypsin-like activity of the proteasome. The activity was determined by monitoring the accumulation of the fluorescent cleaved product 7-amido-4-methylcoumarin, using a temperature-controlled automatic multiwell plate reader (BioTek Synergy Spectrophotometer, Wynooski, VT, USA) at excitation/emission wavelengths of 380/460 nm. Fluorescence was measured every 5 min during 1 hour, linear rates calculated as fluorescence intensity per second per microgram of protein (ΔFU/s/μg protein) and expressed as percentage of control samples.

4.3.4 Western blot analysis

Mice midbrain and *striatum* were dissected and tissue extracts were prepared as previously described (Castro-Caldas et al. 2009b). Samples were resolved by 10% SDS-PAGE and transferred onto Imobilon P membrane. Membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0,1% Tween-20, for 1 hour at room temperature and further probed with antibodies to ubiquitin (1:1000) or E1 (1:1000), overnight at 4ºC, followed by incubation with secondary antibodies, for 1 hour at room temperature. Analysis of β-actin (1:15000) expression was done in parallel as a loading control. The immunocomplexes were detected by the ECL chemiluminiscent method and visualized with Hyperfilm ECL. Results were quantified using the Gel-Pro Analyzer densitometry analysis software (Media Cybernetics, MD, USA).

4.3.5 Preparation of ^{125}I-labeled ubiquitin

Ubiquitin was radio-iodinated as described previously (Jahngen et al. 1986; Marques et al. 2006). Briefly, ubiquitin was dissolved in 50 mM Tris-HCl pH 7.6 and mixed with Na^{125}I. The iodination reaction was initiated by addition of chloramine T, and the vials were shaken for 2 min at room temperature. The reaction was terminated by the addition of sodium meta-bisulfite and sodium iodide. Free ^{125}I and small protein fragments were removed from the protein solution by Centricon-10 micro-concentrators.

4.3.6 Determination of ubiquitin conjugation activity

In order to determine the conjugation capacity of the UPS components from midbrain and *striatum* tissue extracts to catalyze the conjugation of exogenous radio-labeled ubiquitin (^{125}I-Ub) to endogenous protein substrates a modification of an assay described by Hersko and *col*. 1980 (Hershko et al. 1980) was used. Mice midbrain and *striatum* were dissected and homogenized in 50 mM Tris-HCl pH 7.6 and sonicated at 4ºC. The soluble fraction obtained after centrifugation at 15000*g*, at 4ºC, for 15 min was used to perform the assay and its protein content was determined by the Coomassie method.

The conjugation activity assay was performed in a final volume of 25 μL, containing 50 μg of tissue extract and 10 μL of conjugation buffer (50 mM Tris-HCl,

pH 7.6, 1 mM DTT, 5 mM $MgCl_2$, 34.8 U/mL creatine phosphokinase, 10 mM creatine phosphate, 2 mM ATP and 0.3 μg of ^{125}I-Ub (approximately $2x10^5$ cpm)). Following 30 min of incubation at 37ºC, the reaction was stopped by addition of 25 μL of Laemmli buffer. After incubation at room temperature for 30 min, the proteins were separated by 12% SDS-PAGE. The formation of *de novo* ubiquitin conjugates was visualized by autoradiography of dried gels.

4.3.7 Determination of ubiquitin-activating and conjugating enzymes activities –thiol ester assay

This assay was carried out as described above for the ubiquitin conjugation assay except that the incubation period was 5 min at 37ºC and the reaction was stopped by addition of 25 μL of Laemmli buffer or thiol ester assay buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 8 M urea, 10% glycerol and 0.1 mg/mL bromophenol blue). After incubation at room temperature for 30 min the proteins were separated by SDS-PAGE on 15% gels. The activities of E1 and E2 were quantified by determining the density of the bands on the autoradiogram of dried gels. Samples treated with β-mercaptoethanol were used as negative controls for the reactions.

4.3.8 Statistical analysis

Statistical analyses were performed using the software Graphpad Prism 5.0 (SanDiego, CA, USA). Data were statistically evaluated for significance using ANOVA with Tukey post-hoc test for multiple comparisons. Differences were considered statistically significant when $p<0.05$.

4.4 Results

4.4.1 Impairment of proteasome activity by MPTP and MG132 in mice midbrain and *striatum*

In order to clarify the involvement of proteolytic system impairment in the dopaminergic neuronal damage caused by MPTP-induced oxidative stress we assessed the function of several UPS constituents. We started by measuring the three peptidase

activities of the proteasome in wt and GSTP ko mice in both midbrain and *striatum* regions, at 6 and 24 h post-treatment.

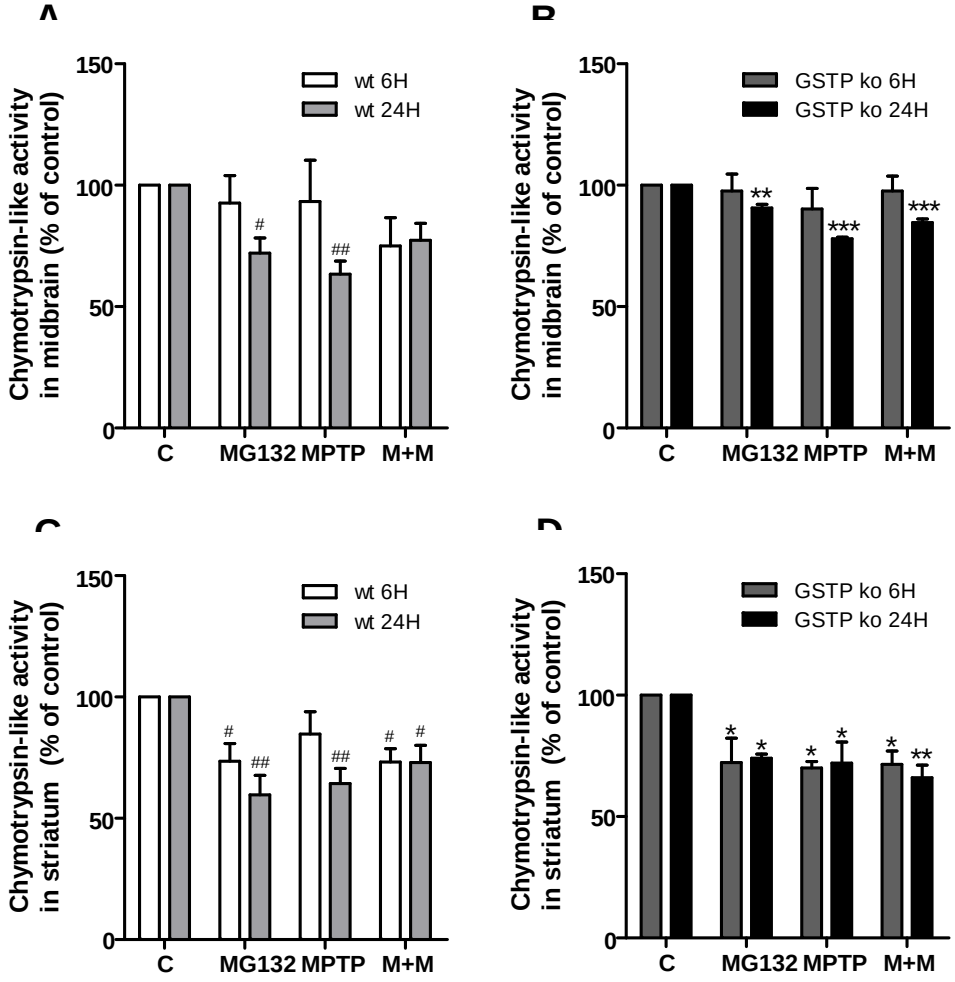

Figure 4.1. Proteasome activity in mice midbrain and *striatum* upon MPTP and MG132 administration. Tissue extracts were prepared from midbrains and *striata* from C57BL/6 wild-type and GSTP ko mice i.p. injected with saline (control), MPTP (40mg/kg) and/or MG132 (5mg/kg), and sacrificed 6 or 24 hours post-treatment. Proteasome chymotrypsin-like activity in the midbrain (**A**, **B**) and *striatum* (**C**, **D**) from wild-type (**A**, **C**) and GSTP ko (**B**, **D**) was determined using the fluorogenic synthetic peptide substrate Suc-LLVY-AMC. The increase in fluorescence intensity (ΔFU) of the enzymatically cleaved product was detected at excitation/emission wavelengths of 380/460 nm. Results were obtained as ΔFU/s/µg protein and then converted to mean percentage activity. Chymotrypsin-like activity in control samples was arbitrarily set as 100% and its levels in MPTP and/or MG132-treated samples were plotted as percentage of this value. Data presented are mean triplicate values ± SEM of at least three independent experiments. Statistical analysis was carried out using one-way ANOVA with Tukey post-hoc test, where $^{\#}p<0.05$, $^{\#\#}p<0.01$, relative to wt control and $^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$ relative to GSTP ko control.

We observed significant decreases in the chymotrypsin-like activity of the proteasome in both midbrain (Figure 4.1 A and B) and *striatum* (Figure 4.1 C and D) of wt (Figure 1 A and C) and GSTP ko mice (Figure 4.1 B and D). In the midbrain of both wt and GSTP ko mice the decrease in this activity was only significant at 24 h after the administration of MPTP or MG132 (Figure 4.1 A and B). In the *striatum* of wt mice chymotrypsin-like activity was decreased by approximately 30% at 6 h ($p<0.05$) and 40% at 24 h ($p<0.01$), in MG132-treated mice (Figure 4.1 C). MPTP in turn, led to a 35% ($p<0.01$) decrease in chymotrypsin-like activity at 24 h post-injection (Figure 4.1 C). The concomitant administration of MPTP and MG132 led to a decrease of approximately 30% ($p<0.05$) in this proteolytic activity at both 6 h and 24 h post-injection (Figure 4.1 C). Administration of MPTP or MG132 resulted in inhibition of the chymotrypsin-like activity, in the *striatum* of GSTP ko mice, of approximately 30% ($p<0.05$), at both time-points analyzed (Figure 4.1 D). Simultaneously administration of MG132 and MPTP reduced the proteasomal activity by approximately 30% at 6 h ($p<0.05$) and 40% at 24 h ($p<0.01$) (Figure 4.1 D). No significant changes, in neither trypsin-like nor peptidylglutamyl peptide hydrolase activities, were detected (data not shown).

4.4.2 Endogenous ubiquitin-protein conjugates levels are altered in response to proteasome inhibition and MPTP-induced oxidative stress

The levels of endogenous ubiquitin conjugates at a given time-point result from the balance between the ubiquitin conjugation activity and the degradation of the ubiquitin conjugates by the proteasome or deubiquitination by isopeptidases. In order to further assess the effect of MG132 and MPTP-induced oxidative stress on the ubiquitin-proteasome pathway we determined the levels of endogenous ubiquitin conjugates accumulated in the midbrain and *striatum* of wt and GSTP ko mice injected with these compounds.

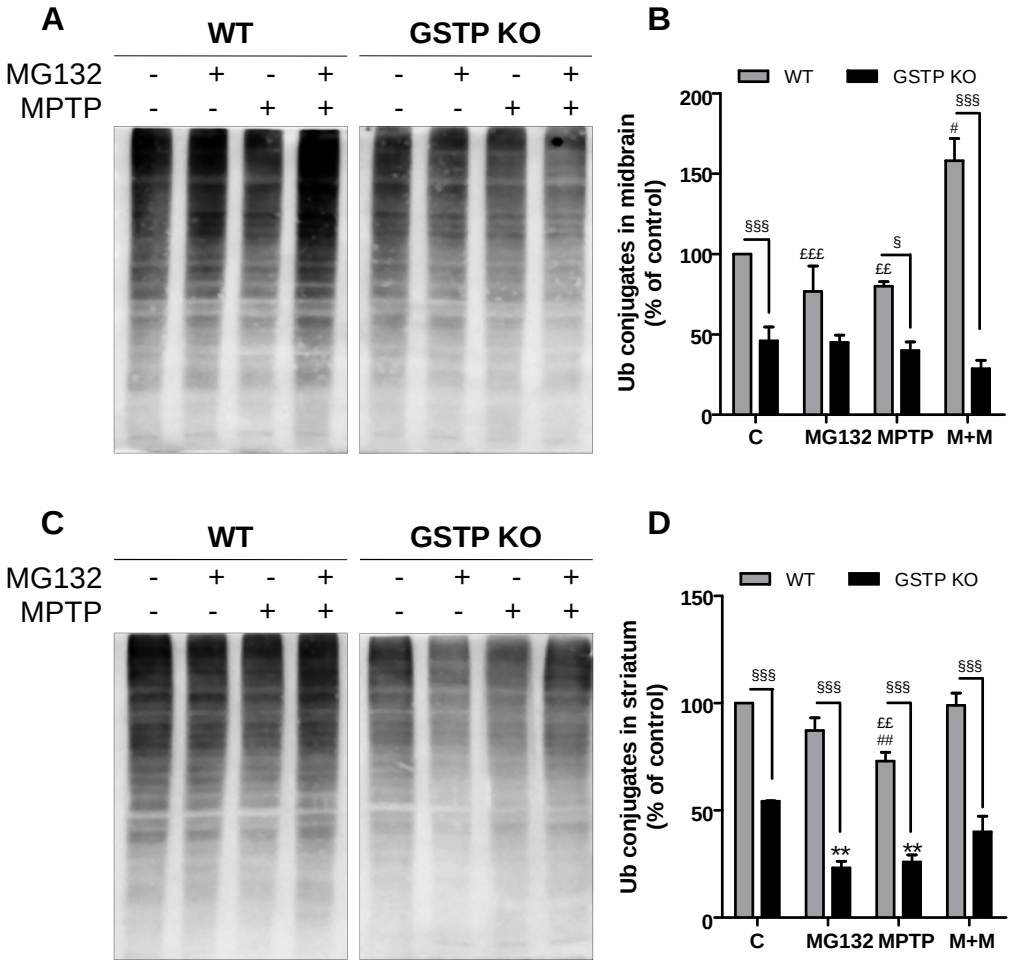

Figure 4.2. Ubiquitin conjugates levels in the midbrain and *striatum* of MPTP and MG132-treated C57BL/6 wild-type and GSTP ko mice. Six hours after i.p. administration of saline (control), MG132 (5 mg/Kg) and/or MPTP (40 mg/Kg), mice were sacrificed and brains were dissected. Endogenous ubiquitin conjugates levels were determined in midbrain (**A, B**) and *striatum* (**C, D**) from wild-type and GSTP ko mice, by Western blot analysis, using a mouse anti-ubiquitin antibody. The ubiquitin (Ub) conjugates levels in control samples, from saline-treated wt mice, were arbitrarily set as 100% and levels in MPTP and/or MG132-treated samples were calculated and plotted as a percentage of this value. Data shown are mean ± SEM of at least three independent experiments. Statistical analysis was carried out using one-way ANOVA with Tukey post-hoc test where $^{\#}p<0.05$, $^{\#\#}p<0.01$, relative to wt control; $^{\pounds\pounds}p<0.01$, $^{\pounds\pounds\pounds}p<0.001$, relative to wt MG132 plus MPTP; $^{**}p<0.01$, relative to GSTP ko control; and two-way ANOVA with Bonferroni's post-hoc test where $^{\S}p<0.05$, $^{\S\S\S}p<0.001$ wild-type *vs* corresponding GSTP ko.

GSTP ko treated with MG132, MPTP or with the combination of MG132 plus MPTP display significantly lower levels of endogenous ubiquitin conjugates accumulation when compared to their wt counterparts, in both midbrain (Figure 4.2 A and B) and *striatum* (Figure 4.2 C and D). However, for GSTP ko mice, only in the *striatum*

the treatments with MG132 or with MPTP induced a significant ($p<0.01$) reduction in the levels of the endogenous ubiquitin conjugates (Figure 4.2 D). The accumulation of ubiquitin conjugates in wt mice, in turn, is significantly affected by the treatment with both compounds, that induced a significant ($p<0.05$) increase in accumulation of ubiquitin conjugates in the midbrain (Figure 4.2 B).

4.4.3 MPTP and MG132 induce alterations in ubiquitin conjugation capacity in mice midbrain and *striatum*

To assess the function of the enzymes that constitute the endogenous ubiquitin conjugation machinery, the ability to form *de novo* ubiquitin conjugates was evaluated in tissue extracts from both wild-type and GSTP ko mice, using exogenous radio-labeled ubiquitin (^{125}I-ubiquitin).

As shown in Figure 4.3, we detected a significant decrease in the formation of conjugates of ubiquitin with protein substrates in the midbrain of wt mice treated with MG132 ($p<0.05$) or with MPTP ($p<0.01$) (Figure 4.3 A and B). Similarly the co-administration of both compounds led to a 55% ($p<0.01$) decrease in the *de novo* formation of ubiquitin conjugates (Figure 4.3 A and B). In GSTP ko mice, after injection of both compounds, the conjugation activity in the midbrain is lowered by approximately 60% ($p<0.05$) (Figure 4.3 A and B).

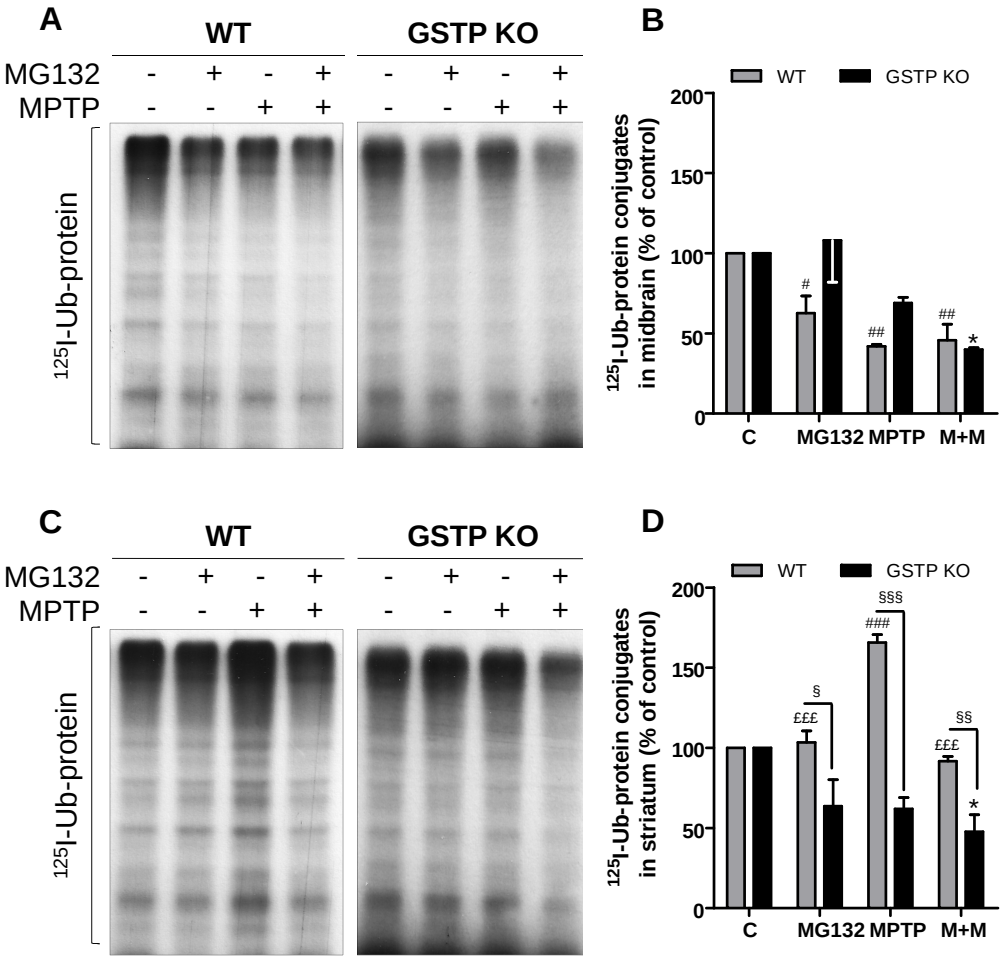

Figure 4.3. Ubiquitin conjugation activity in C57BL/6 wild-type and GSTP ko mice midbrain and *striatum* upon treatment with MPTP and MG132. C57BL/6 mice were i.p. injected with saline (control), MG132 (5 mg/kg) and/or MPTP (40mg/kg), euthanized 6 hours after treatment and midbrains and *striata* were dissected. Ubiquitin conjugation activity was determined by incubation of tissue extracts with exogenous ^{125}I-ubiquitin followed by SDS-PAGE analysis. The gels were stained, dried, and subjected to autoradiography. The amount of *de novo* ubiquitin conjugates formed represents ubiquitin conjugation activity in the midbrain (**A, B**) and *striatum* (**C, D**) of wild-type and GSTP ko mice. The autoradiographs shown are representative of three independent experiments. The ubiquitin conjugates levels in control samples were arbitrarily set as 100% and the relative levels in MPTP and/or MG132-treated samples was calculated and plotted as a percentage of this value. Data shown are mean ± SEM of three independent experiments. Statistical comparisons were performed using one-way ANOVA with Tukey post-hoc test where $^{\#}p<0.05$, $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ relative to wild-type control; $^{*}p<0.05$, relative to GSTP ko control; $^{£££}p<0.001$, relative to MPTP-treated wild-type; $^{§}p<0.05$, $^{§§}p<0.01$, $^{§§§}p<0.001$wild-type *vs* corresponding GSTP ko.

GSTP ko mice exhibited similar results in both brain regions with a 50% decrease ($p<0.05$) in the conjugating activity in the *striatum* of MG132 plus MPTP treated mice (Figure 4.3 C and D). However, surprisingly in the *striatum* of wt mice the administration of MPTP resulted in a significant increase (~65%, $p<0.001$) in the ability to form *de novo* conjugates (Figure 4.3 C and D).

Moreover, in the *striatum*, the alterations in the conjugation activity levels were significantly different in GSTP ko mice when compared to their wt counterparts under the same treatment schedule (Figure 4.3 C and D).

4.4.4 Ubiquitin-activating enzyme expression and activity is modified by MPTP and MG132 treatments in mice midbrain and *striatum* of wild-type and GSTP knock-out mice

E1 is the rate-limiting enzyme in the ubiquitin conjugation process and its activity has been suggested to be transiently up-regulated in response to oxidative stress (Shang et al. 1997). In accordance with the observed significant increase in the *de novo* formed conjugates in the *striatum* of wt mice upon MPTP administration (Figure 4.3 C and D), the E1 activity in this brain region is also up-regulated in mice treated with the neurotoxin (Figure 4.4 C). Furthermore, in the midbrain of wt mice (Figure 4.4 A) and in both the midbrain (Figure 4.4 B) and *striatum* (Figure 4.4 D) of GSTP ko mice the levels of E1~Ub thiolester are lowered by the treatment with MG132 plus MPTP, which is also in accordance with the data obtained for the *de novo* ubiquitin conjugates levels in these same brain regions (Figure 4.3).

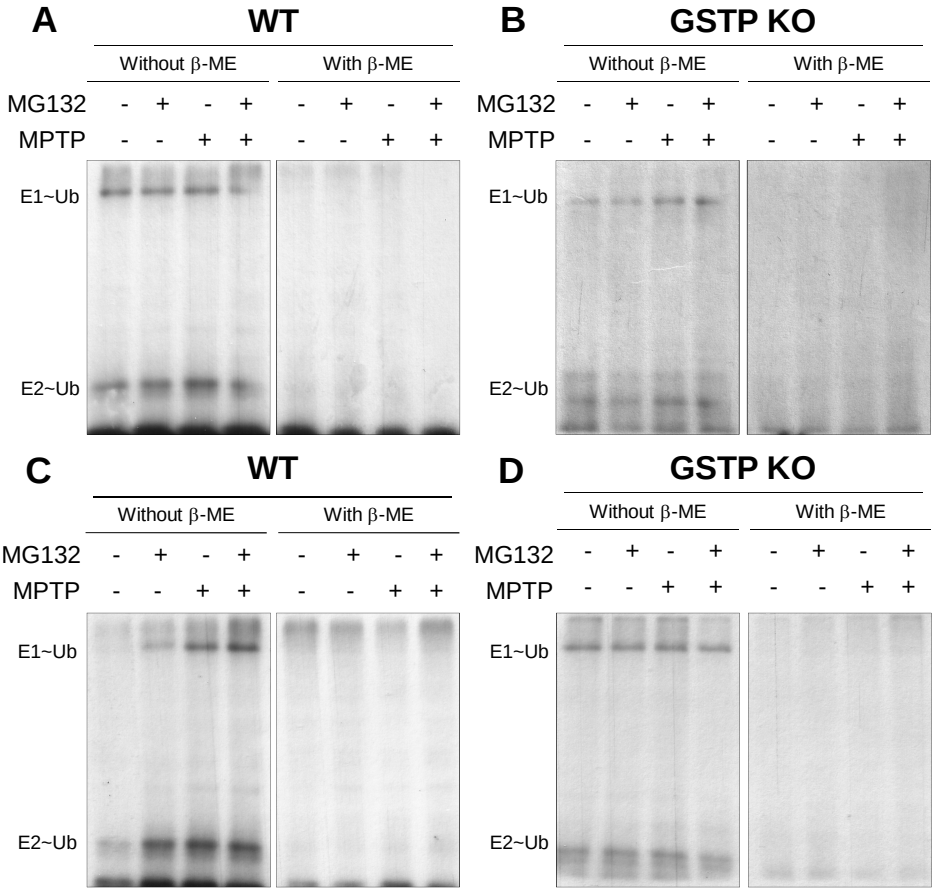

Figure 4.4. Ubiquitin activating enzyme (E1) and ubiquitin conjugating enzymes (E2s) activities in response to MPTP and MG132 administration. Tissue extracts from C57BL/6 wild-type (**A**, **C**) and GSTP ko (**B**, **D**) mice midbrains (**A**, **B**) and *striata* (**C**, **D**) were prepared after saline (control), MPTP (40mg/kg) and/or MG132 (5mg/kg) i.p. injection, and sacrificed 6 hours post-treatment. Ubiquitin activating enzyme (E1) and ubiquitin conjugating enzyme (E2) activities following MPTP/MG132 treatment were determined by thiolester assay using exogenous ^{125}I-ubiquitin. In parallel, as a control for this assay, tissue extracts were exposed to β-mercaptoethanol (β-ME) because E1~Ubiquitin (E1~Ub) and E2~Ubiquitin (E2~Ub) thiolesters are labile to reducing agents. The bands that are not visible in the presence of β-ME are E1~Ub and E2~Ub thiolesters.

Moreover, the E1 protein levels are significantly lower in GSTP ko mice when compared to wt mice under proteasome inhibition or treatment with the neurotoxin, in both brain compartments (Figure 4.5 B and D). Additionally, whereas in the *striatum* of wt mice E1 protein levels increase in response to the insults, in GSTP ko mice E1 expression is decreased upon exposure to both compounds (Figure 4.5).

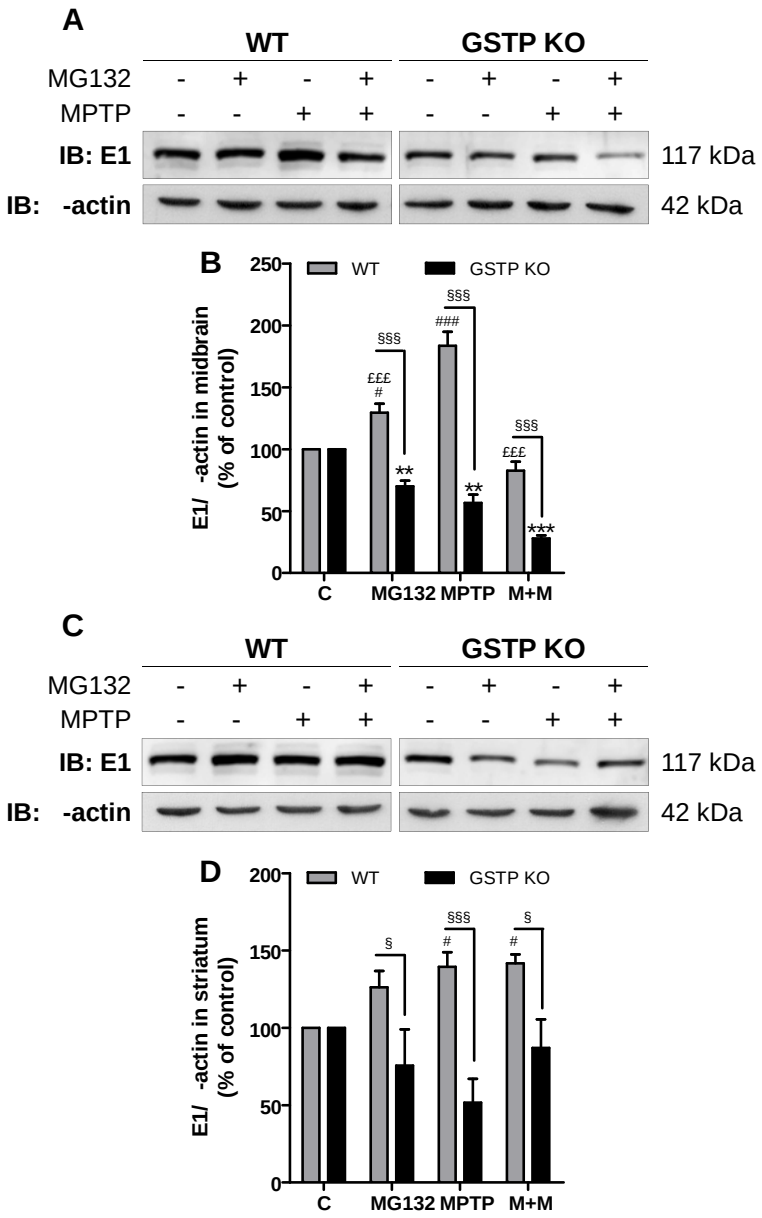

Figure 4.5. Ubiquitin activating enzyme (E1) expression levels in response to MPTP and MG132 administration. C57BL/6 wild-type and GSTP knockout mice were i.p. injected with saline (control), MPTP (40 mg/Kg) and/or MG132 (5 mg/Kg) and sacrificed 6 hours post-treatment. Tissue extracts were prepared from midbrain **(A**, **B)** and *striatum* **(C**, **D)** from both wt and GSTP ko mice and subjected to SDS-PAGE. The corresponding blots were probed with a rabbit anti-E1 antibody. Analysis of β-actin was done in parallel as a loading control. The relative levels of E1 in control samples were arbitrarily set as 100% and the relative levels in MPTP and/or MG132-treated samples were calculated and plotted as a percentage of this value. Data shown are mean ± SEM of at least three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test where $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ relative to wild-type control; $^{**}p<0.01$, $^{***}p<0.001$, relative to GSTP ko control; $^{£££}p<0.001$, relative to MPTP-treated wild-type; and two-way ANOVA with Bonferroni's post-hoc test where $^{§}p<0.05$, $^{§§§}p<0.001$wild-type *vs* corresponding GSTP ko.

4.5 Discussion

In sporadic PD, neuronal cell death is accompanied by the accumulation of misfolded protein deposits in affected brain regions (Dauer and Przedborski 2003), suggesting that a failure in the cellular protein degradation pathways might contribute to the pathogenesis of the disease (Ciechanover and Brundin 2003; Olanow and McNaught 2006).

The selective removal of oxidative-damaged proteins by the UPS is essential for the cells to survive under conditions of environmental stresses (Jahngen-Hodge et al. 1997; Dudek et al. 2005). However the UPS is itself a target of oxidative stress and it has been previously reported that different components of the UPS have different susceptibilities to oxidative stress (Zhang et al. 2008).

Oxidized proteins are generally preferred proteasome substrates (Davies 2001; Grune et al. 2003) and upon oxidizing conditions the cell can initiate a response that involves the upregulation of both proteasome activity and ubiquitin conjugation enzymes leading to enhanced intracellular protein degradation. However, due to the different susceptibilities to oxidative stress, proteasome can be inactivated and even so the ubiquitin conjugation machinery may still function properly. In this scenario proteins are tagged for degradation by ubiquitin but are not degraded by the proteasome resulting in an accumulation of ubiquitin conjugates. On the other hand, extensive oxidative stress that compromises both the proteasome and the ubiquitination enzymes results in a decrease in the *de novo* synthesis of ubiquitin-protein conjugates and in proteasomal degradation (Shang and Taylor 2011).

The different susceptibilities of the UPS components dictate the UPS response to various levels of oxidative stress. The proteasome has been reported to be more susceptible to oxidative stress-induced inactivation than the enzymes of the ubiquitin conjugation machinery (Shang et al. 1997; Zhang et al. 2008).

We have previously observed that both MPTP and MG132 *in vivo* administration induces oxidative stress as demonstrated by increased levels of reactive oxygen species and protein carbonyls and decreased glutathione levels (Neves Carvalho *et al* unpublished results, see chapter 3 of this thesis). We have also shown that GSTP protects against MPTP-induced neurotoxicity by modulating JNK activity (Castro-Caldas et al. 2012) and the Nrf2 antioxidant pathway (Neves Carvalho *et al* unpublished results, see chapter 3 of

this thesis) in a mouse model of PD. Here, using a similar experimental paradigm of MG132 and MPTP treatments in wild-type and GSTP ko mice we investigated the effect of MPTP-induced oxidative stress on the UPS function, in mice midbrain and *striatum*.

Our present results show that MG132 and MPTP affected the proteasome proper function leading to a decrease in the chymotrypsin-like activity in both wild-type and GSTP ko mice. The proteasome function seems to be altered by the treatments in a similar manner in both wt and ko mice, although in GSTP ko mice treated with MPTP the inhibition of the proteasome in the *striatum* occurs at the earlier time-point tested when compared to wt mice.

Oxidative damage to the proteasome is one possible mechanism that enhances stress-induced accumulation of ubiquitin conjugates in the cells. Previous reports indicate that the levels of endogenous ubiquitin conjugates are a sensitive marker of oxidative stress (Shang and Taylor 1995; Figueiredo-Pereira et al. 1997; Shang et al. 1997; Adamo et al. 1999). However, whereas proteasome activity and UPS function might be upregulated by mild oxidative stress, leading to ubiquitin conjugates accumulation; severe oxidative stress could impair proteasome activity and UPS function in fact reducing the levels of ubiquitin conjugates (Zhang et al. 2008; Shang and Taylor 2011).

In the present study we observed a significant decrease in the levels of endogenous ubiquitin conjugates in the *striatum* of both wild-type and GSTP ko mice in response to MPTP administration, pointing towards a severe effect of acute MPTP administration in the physiological / steady-state function of the UPS. Notably, ubiquitin-conjugates levels of both control and MG132/MPTP treated mice were significantly lower in GSTP ko mice when compared with their wt counterparts. Additionally, ubiquitination capacity of GSTP ko mice in the *striatum* is also significantly decreased when compared to the wild-type mice. These results indicate that the UPS is more affected by oxidative stress in GSTP ko mice than in wt mice. Moreover, wt mice have the ability to increase ubiquitination, by inducing E1 activity, as an attempt of the cells to increase the degradation of damaged proteins in response to MPTP-induced oxidative stress.

In accordance, the levels of E1 enzyme, upon treatment with MG132 or MPTP, are also significantly lower in GSTP ko mice when compared to their wt counterparts. Moreover, in GSTP ko mice, treatment with MPTP, MG132 or with both compounds induce significant decreases in midbrain E1 protein levels. In contrast, in wt mice both E1

expression and activity tend to increase upon treatment with MPTP, indicating that wt mice have a higher capacity to cope with MPTP-induced oxidative stress and still maintain a functional UPS.

Taylor and collaborators have proposed that activities of E1 and E2 enzymes are regulated by cellular redox status, namely GSSG/GSH ratio (Obin et al. 1998). Moreover, an independent report (Figueiredo-Pereira et al. 1997) showed that changes in UPS function could be directly related to a decrease in GSH levels and an increase in *S*-glutathionylated proteins.

We have previously reported that MPTP induces a significant decrease in glutathione levels that is accompanied by an increase in *S*-glutathionylated protein levels in C57BL/6 wild-type mice brain (Neves Carvalho *et al* unpublished results, see chapter 3 of this thesis). Interestingly all the enzymes in the ubiquitin conjugation pathway have a cysteine in their active sites, and therefore might be subjected to regulation through *S*-glutathionylation (Jahngen-Hodge et al. 1997). Glutathionylation, being a reversible process, might protect protein cysteine residues from irreversible oxidative-driven inactivation. As we previously observed (Neves Carvalho *et al* unpublished results, see chapter 3 of this thesis) GSTP ko mice exhibit lower global levels of *S*-glutathionylation when compared to wt mice, in response to MPTP and MG132 treatments. This might account for the inability for the upregulation of E1 activity in GSTP ko mice in response to MPTP-induced oxidative stress.

Here we show that GSTP ko mice under MPTP treatment display impaired ubiquitination capacity and lower expression levels of E1, the rate-limiting enzyme in the ubiquitination process. Since the increase in proteolysis may function as an anti-oxidant mechanism, by enhancing the removal of oxidative-damaged proteins from cells, our results strongly suggest that GSTP ko mice exhibit deficits in the UPS, that may contribute to their higher susceptibility to MPTP-induced neurotoxicity. Taken together the present results show that GSTP ko mice are more susceptible to UPS failure, under conditions of oxidative stress and proteasome inhibition, reinforcing our previous data and indicating that GSTP has also a role in protecting protein degradation pathways against oxidative stress.

Acknowledgments

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5

CONCLUDING REMARKS

The increase in life expectancy in the last decades reflects the advances in scientific research, medicine and health care. However, unfortunately, a number of diseases are frequently associated with longer life time, particularly neurodegenerative diseases, deeply decreasing the quality of life of the patients. These diseases, together with the remaining generally called brain disorders, have a high prevalence as well as short- and long-term impairments and disabilities and account for one-third of the burden of all diseases in the wealthy part of the world (Gustavsson et al. 2011; Olesen et al. 2012). Effective translational brain research can alleviate human suffering and have a major impact on economic and health care costs. According to the recommendations of the European Brain Council, brain research is a priority for the next years and funding for this area should be increased (Gustavsson et al. 2011).

Although affecting different regions of the brain and causing diverse disabilities, the vast majority of neurodegenerative diseases are invariably characterized by neuronal loss, and share common pathological features, namely mitochondrial dysfunction, oxidative stress and impairment of protein degradation pathways (Jenner 2003; Henchcliffe and Beal 2008; Schapira and Jenner 2011; Exner et al. 2012; Schapira 2012).

Oxidative damage caused by the generation of reactive oxygen species that is associated with these processes is a central issue in the progression of disease. Therefore, antioxidant therapies are potential promising approaches to counteract ROS-mediated damaged in neurodegenerative disorders in general, and particularly in PD (Schreibelt et al. 2007; Jazwa and Cuadrado 2010; Jazwa et al. 2011; Sureda et al. 2011; Vina et al. 2011). However, a main drawback that limits the development of antioxidant therapies has been that most exogenous antioxidants do not cross the blood-brain barrier. Therefore, compounds that are able to stimulate the endogenous antioxidant machinery will certainly provide good therapeutic candidates.

In order to pursue novel therapeutic agents that target the processes common to all neurodegenerative diseases, an improvement in the knowledge of the molecular mechanisms underlying neuronal cell death and potential protective pathways is required.

The original studies presented in this thesis were mainly aimed at contributing to a better understanding of the pathways involved in dopaminergic neurodegeneration in the MPTP mouse model of PD, as well as in characterize the mechanisms through which GSTP might modulate these pathways to achieve neuroprotection.

We have demonstrated that the sub-acute administration of MPTP to wild-type and GSTP knockout mice induces a progressive loss of nigral dopaminergic neurons together with the degeneration of striatal fibres. DA degeneration occurs earlier in GSTP ko mice showing that GSTP ko mice are more susceptible to the neurotoxic effect of MPTP than their wt counterparts, and suggesting that GSTP might indeed have a protective role against neurotoxicity. Additionally, we observed that MPTP treatment leads to increased phosphorylation of JNK, as well as, an increase in JNK catalytic activity. The activation of the JNK signalling pathway is known to trigger cell death pathways, therefore our results are in accordance with previous studies (Saporito et al. 2000; Gearan et al. 2001; Aoki et al. 2002; Peng and Andersen 2003; Hunot et al. 2004) and indicate that this is one of the pathways involved in DA neuronal demise. The molecular mechanisms underlying the regulation of JNK activity involve the activation of a kinase signalling cascade as well as several endogenous negative regulators of JNK. Our results demonstrate that *in vivo* GSTP modulates the response to MPTP-induced oxidative stress, by acting as an endogenous regulator of JNK activity through protein-protein interactions.

GSTP expression can be induced by one of the major regulators of the cellular antioxidant response, the transcription factor Nrf2. Nrf2 activity is mainly controlled by Keap1, that under physiological conditions drives Nrf2 for proteasomal degradation. However, upon oxidative stress cysteine residues in Keap1, that act as redox sensors, are modified inducing changes in conformation that lead to the disruption of the Keap1-Nrf2 complex and subsequent Nrf2 activation upon translocation to the nucleus.

In the present work, we seek to clarify in what way did the proteasome inhibition, additionally to MPTP, modified Nrf2 activity and consequently the expression of its downstream targets, namely GSTP and HO-1. The results presented herein showed an increase in ROS and protein carbonyls (indicative of the presence of oxidized proteins), as well as astrogliosis and reactive microglia in the brain of wild-type mice treated with MPTP and/or the proteasome inhibitor, MG132. Taken together, these results suggest the early activation of stress and inflammatory pathways, in which seems to exist a synergistic effect for the concomitant administration of both compounds.

These results are in accordance with the previously described effects of increased oxidative stress and neuroinflammation triggered by MPTP (Przedborski et al. 2000; Hunot et al. 2004; McGeer and McGeer 2008; Hirsch and Hunot 2009). Moreover,

proteasome inhibition has also been suggested to recapitulate some of the neuropathological features of DA degenerative process (McNaught et al. 2004) and can thus aggravate the effects of MPTP. Curiously, although UPS impairment has been implicated in idiopathic PD, where decreased proteasome activities where described in the SN of patients (McNaught and Jenner 2001), several studies also suggest that mild impairment of the UPS could have protective effects in cellular and animal models of PD (Inden et al. 2005; Yew et al. 2005). Moderate inhibition of the UPS determines the onset of intracellular protein inclusions (that may or may not be resultant of a protective strategy) whereas increasing dosage leads to cell death (Sawada et al. 2004; Fornai et al. 2005). Indeed, although experimental evidence indicate that neurodegeneration is frequently associated with impaired UPS function, whether this is a cause or a consequence of other events occurring along the pathological process is still controversial.

One of the most well-known markers of the cellular redox status is the amount of intracellular glutathione, as well as its oxidation status. We observed a decrease in glutathione levels, accompanied of increased GPx activity and decreased GR activity, indicating a more oxidizing intracellular milieu. Nevertheless, this was not reflected in an increase of the free oxidized form of glutathione, most likely because the observed decrease in glutathione levels was due to its conjugation with proteins, since we observed an increase in *S*-glutathionylated protein levels in the brain of wt mice treated with MPTP.

Importantly, by co-immunoprecipitation assays we demonstrated for the first time the *in vivo* specific *S*-glutathionylation of Keap1. This post-translational modification is able to alter cysteine residues in Keap1 promoting the dissociation of the Nrf2-Keap1 complex. In accordance, we also observed Nrf2 nuclear translocation by both immunofluorescence confocal microscopy and western blot analysis, and verified its activation through the increase in the expression of its downstream targets, GSTP and HO-1. We verified the presence of *S*-glutathionylated Keap1 in wt mice but not in GSTP ko mice. Moreover, in GSTP ko mice we do not observe an increase in HO-1 levels, indicating that there was no activation of Nrf2. Since GSTP has been suggested to promote *S*-glutathionylation following oxidative and nitrosative stress (Townsend et al. 2009a), these results strongly suggest that in response to MPTP-induced oxidative stress, GSTP enables Keap1 *S*-glutathionylation, leading to dissociation of the Keap1-Nrf2

complex and subsequent Nrf2 activation. Nrf2 is translocated to the nucleus where it enhances the expression of several cytoprotective genes, including GSTP and HO-1. The fact that we did not observe this mechanism in GSTP ko mice strongly indicates that GSTP has indeed a role in this process. Therefore, by this way GSTP is able to regulate its own expression through a positive feedback mechanism. This constitutes a further self-regulatory mechanism adding to the already described *S*-glutathionylation of GSTP itself (Townsend et al. 2009a).

Overall these results demonstrate that MPTP induces early significant changes in the redox status in the mouse brain. In response to this insult the main cellular antioxidant mechanisms, namely those regulated by Nrf2, are triggered. GSTP plays a role in the regulation of this process by enabling Keap1 S-glutathionylation in a feedback regulatory loop. Furthermore, when the UPS is impaired (by concomitant administration of the proteasome inhibitor) cells are no longer able to mount an adequate antioxidant protective response against the MPTP insult. Our results further strengthen the hypothesis of the impairment of protein degradation pathways as a contributing factor in the neurodegenerative processes and unravel an additional molecular mechanism contributing to GSTP-elicited neuronal protection.

Dysfunction of protein degradation pathways is one of the contributing factors for the DA neuronal degeneration. The impairment of protein degradation pathways, namely the proteasome, could cause the accumulation of damaged, oxidized proteins. On the other hand, the accumulation of oxidized proteins could lead to the overload and consequent inhibition of the proteasome, creating a vicious cycle that potentiates oxidative damage to the cells.

In the final part of this work we sought to elucidate the effect of both MPTP and MG132 in the function of the UPS and to clarify if the absence of GSTP alters the response of this pathway to the neurotoxin and proteasome inhibitor insults. We could observe that, similarly to what have been described in other models (Zhang et al. 2008; Shang and Taylor 2011), in our model different components of the UPS display different susceptibilities to oxidative stress. Furthermore, importantly we verified that GSTP ko mice are more susceptible to UPS dysfunction, upon MPTP administration, when compared to their wt counterparts, displaying decreased ubiquitination capacity and lower levels of the ubiquitin-activating enzyme. Given that the increased proteolysis can function as an antioxidant mechanism, by degrading oxidized, dysfunctional proteins, and

thus preventing their accumulation, the fact of GSTP ko mice being less resistant to potential damages in this pathway could contribute to their increased susceptibility to MPTP-induced oxidative stress. These results also suggest that GSTP could have a role in the protection and maintenance of functional protein degradation pathways in oxidative stress conditions. Additionally, the enzymes of the ubiquitination machinery where shown to be regulated by redox cellular status, namely GSH and GSSG levels (Jahngen-Hodge et al. 1997; Obin et al. 1998), and the activity of the ubiquitin-activating enzyme was shown to be transiently up-regulated upon oxidative stress conditions (Shang et al. 1997). Moreover, the *S*-glutathionylation status of a protein can determine their preferable degradation by the proteasome (Zetterberg et al. 2006). Furthermore, all the enzymes of the ubiquitination machinery possess a cysteine residue in their active centre that can be targeted for *S*-glutathionylation. Therefore, it is plausible to presume that these enzymes could be regulated by this post-translational modification and possibly by GSTP.

In conclusion, the results presented in this thesis clarify some of the mechanisms and pathways involved in dopaminergic neuronal degeneration and provide a significant contribution to the understanding of the molecular mechanisms underlying GSTP-elicited neuronal protection *in vivo*. GSTP altered expression and function has been associated with several diseases as disparate as malignancies and neurodegenerative diseases. Our results show that besides an important role in detoxification, *in vivo* GSTP has the ability to regulate JNK catalytic activity, therefore modulating cell death pathways, as well as to enable the *S*-glutathionylation of important regulators of the antioxidant response.

Therefore, as a central player in the endogenous antioxidant response and due to its important catalytic and non-catalytic functions, GSTP may prove to be a privileged therapeutic target in the development of novel combined therapeutic strategies that can effectively target multiple aspects of the intricate molecular pathology of Parkinson's disease and other neurodegenerative disorders.

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